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The Effect of WDR1 and a Spliced Isoform (WDR Δ 35) on Cell Migration in Mammalian Cells

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**The Effect of WDR1 and a Spliced Isoform (WDR Δ 35) on Cell Migration in
Mammalian Cells**

By

Nicoletta Harabor

A Thesis

Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

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2014

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The Effect of WDR1 and a Spliced Isoform (WDR Δ 35) on Cell Migration in Mammalian Cells

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January 20, 2014

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ABSTRACT

WDR1 is a highly conserved regulator of the actin cytoskeleton in eukaryotes. A novel splice variant of WDR1 has been discovered, WDR Δ 35, which lacks exons 3-5. To determine if functional differences exist between these two isoforms and whether perturbation of WDR1/WDR Δ 35 levels affects the rate of cell migration, they were overexpressed as GFP fusion proteins in HEK293 cells and the rates of cell migration were quantified. Overexpression of WDR1/WDR Δ 35 caused a significant decrease in cell migration compared to the GFP control. The ratio of G/F-actin was measured upon WDR1/WDR Δ 35 overexpression and it was found to be significantly higher for GFP-WDR/ WDR Δ 35 transfected cells compared to GFP alone which may indicate higher actin turnover rates in these cells. Site directed mutagenesis generated several mutants for WDR1/ WDR Δ 35 which indicated that specific residues (e.g. WDRR17G, WDR Δ 35H48Q and WDR Δ 35G204E) could alter the ratio of G/F-actin in cells, suggesting an important structural role in WDR1 function.

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LIST OF ABBREVIATIONS

ABP-	actin binding protein
AC-	actin- depolymerizing factor /cofilin
ADF-	actin- depolymerizing factor
ADP-	adenosine diphosphate
AIP1-	actin- interacting protein 1
ARP-2/3-	actin- related protein-2/3
ATP-	adenosine triphosphate
cAMP-	cyclic adenosine monophosphate
CAP-	adenylyl cyclase-associated protein
Co-IP-	co-immunoprecipitation
CP-	capping protein
C-terminal-	carboxyl terminal
DNA-	deoxyribonucleic acid
ECM-	extracellular matrix
F-actin-	filamentous actin
FH-	formin homology
G-actin-	globular actin
GFP-	green fluorescent protein
HEK293 cells-	human embryonic kidney 293 cells
Hela cells-	cervical cancer cells
kDa-	kilodalton
LAK cells-	Lymphokine-activated killer cells
LIMK-	LIM-kinase
MEK-	MAP (Mitogen-activated protein) kinase or ERK (extracellular signal-regulated kinase)

NHE1- Na^+ - H^+ exchanger
NPF- nucleation promoting factor
N-terminal- amino terminal
OGT- *O*-GlcNAc transferase
O-GlcNAc- *O*-linked N-acetylglucosamine
PAGE- polyacrylamide gel electrophoresis
PBS- phosphate buffered saline
PCR- polymerase chain reaction
PIP2- phosphatidylinositol-4,5-phosphate
RNA- ribonucleic acid
SCAR- suppressor of cyclic AMP repressor
SDS- sodium dodecyl sulphate
siRNA- small interfering RNA
TESK- Testicular protein kinase
TTBS- tris buffered saline + tween
VCA- verprolin homology, central and acidic
WASP- Wiskott-Aldrich Syndrome protein
WAVE- WASP-family verprolin-homologous protein
WDR1- Tryptophan Aspartate Repeat protein 1
WH2- Wiskott-Aldrich syndrome protein homology 2

CHAPTER 1

INTRODUCTION

The actin cytoskeleton

The actin cytoskeleton is an intricate cellular structure in which its assembly and disassembly are involved in many important biological processes such as cell migration, endocytosis and cytokinesis (Kato, 2008). The primary building block of the cytoskeleton is actin, a 43kDa protein that possesses a highly conserved amino acid sequence between all eukaryotic species. It is unique to eukaryotes and exists in α , β and γ isoforms (Dos Remedios, 2003; Sheterline, 1998). In the cell, globular actin monomers (G-actin) assemble to form double-stranded helical polymers of actin filaments (F-actin) which further amass into complex structures like actin bundles and orthogonal networks (Milligan, 1990; Thomas, 2012). Furthermore, each actin filament has a fast growing plus end or barbed end and a slower growing minus end or pointed end due to a structural and kinetic polarity (Pollard, 2003). Actin polymerization is an ATP-dependent process in which each actin monomer contains a cleft that can bind one adenosine nucleotide such as ATP or ADP (Kinosian, 1991). Polymerization first occurs when an actin molecule bound to ATP is added to the barbed end of a filament. Next, actin along with the cofactor Mg^{2+} catalyze the fast hydrolysis of ATP to form an intermediate actin-ADP-Pi molecule, which later loses its Pi at a much slower rate becoming ADP-actin (Yao, 2001; Murakami, 2010). Actin bound to ADP is far less stable than actin-ADP-Pi which allows older filaments to be disassembled at a faster rate than newer filaments (Carrier, 1986). Furthermore, ATP possesses a higher affinity for G-actin than ADP which results in a process called treadmilling where there is a net assembly of actin monomers at the barbed

end and a net disassembly at the pointed end of a filament. Overall, this process can occur at steady state in which the polymer remains at a constant length (Neidl, 1979; Hill, 1982).

During cell migration actin filaments assemble at the cell's leading edge, forming protrusive structures which help drive the cell in a specific direction (Pollard, 2003).

These dynamic cellular features include lamellipodia which are sheet-like membrane protrusions that form at the leading edge of migrating cells and are important in propelling a cell towards a particular destination (Chhabra, 2007; Pollard, 2000). Also, filopodia are thin finger-like protrusions that extend out from the lamellipodia at the front edge of a mobile cell and play an important role in sensing and responding to attractive or repulsive cues in their surrounding environment (Yang, 2011; Chhabra, 2007).

Furthermore, invadosomes are comprised of invadopodia in cancer cells and podosomes in monocytic cells. These structures contain an actin rich core and have the ability to adhere to and degrade the extracellular matrix (ECM) which thus allows them to invade their target cell (Cervero, 2012; Chhabra, 2007). Moreover, in order for cytokinesis to occur in a dividing cell, an actin and myosin based contractile structure assembles and forms a cleavage furrow which causes the cell to ingress and is then disassembled at a later stage (Kato, 2008; Glotzer, 2001). Because of the importance of the actin cytoskeleton in a wide variety of biological processes it is regulated by many different proteins that are involved in nucleation, elongation, severing, crosslinking and stabilizing F-actin in a cell (Thomas, 2012; Pollard, 2000; Winder, 2005).

The main purpose of this study was to investigate and learn more about how one of these actin regulating proteins functions in mammalian cells. The protein that served as the

focus for my research project was mammalian WDR1 (Tryptophan Aspartate Repeat protein 1) which has also been termed Actin Interacting Protein 1 (AIP1) in lower eukaryotes. Furthermore, we have discovered a spliced variant of WDR1 (termed WDR1 Δ 35) which lacks exons 3-5. Both the full length and spliced variant of WDR1 were used for this study. The main hypothesis of this thesis was:

Disruption of WDR1 and WDR Δ 35 levels will affect cell migration in mammalian cells.

Objective 1) Construct and analyze specific mutations within WDR1 and WDR Δ 35 to define potential active functional domains and how it impacts cell movement.

Objective 2) Investigate any differences in functional roles of WDR1 and WDR Δ 35 in mammalian cells through measuring the ratio of F/G actin in cells overexpressing wild type and mutant forms of WDR1 and WDR Δ 35.

Regulators of the actin cytoskeleton

Actin nucleators

Nucleation is one of the first steps involved in generating an actin filament in which an actin trimer known as a nucleus is assembled in the cell. However, these actin dimers and trimers are unstable making spontaneous nucleation a kinetically unfavorable step in actin polymerization (Goley, 2006; Pollard, 2003). Thus, in order to overcome this rate limiting step in F-actin formation a group of proteins known as nucleators, which

include the actin- related protein-2/3 (ARP2/3) complex, formins and spire, act to initiate actin filament assembly through various mechanisms within the cell (Goley, 2006).

Arp2/3 complex

Firstly, the Arp2/3 complex is a protein that is made up of seven polypeptides which include five ARPC1-5 subunits as well as two actin-related proteins called ARP2 and ARP3 which are hypothesized to act as an actin like heterodimer in the initiation of a new actin filament (Bugyi, 2010; Suraneni, 2012; Robinson, 2001). The Arp2/3 complex is inactive by itself and must be stimulated by nucleation promoting factor (NPF) proteins which include members of the Wiskott-Aldrich Syndrome protein (WASP) family and suppressor of cyclic AMP repressor (SCAR) also known as WASP-family verprolin-homologous protein (WAVE) (Goley, 2006; Smith, 2013). Upon binding of the verprolin homology, central and acidic (VCA) region of WASP or SCAR/WAVE to the Arp2/3 complex and a G-actin monomer, Arp2 and Arp3 subunits are brought in closer proximity to one another forming a trimer with actin which nucleates the formation of a new filament from the side of a pre-existing filament (Goley, 2006; Bugyi, 2010; Smith, 2013). Overall, this generates an orthogonal network of actin filaments which are required for lamellipodia extension during cell migration (Chhabra, 2007; Suraneni, 2012).

Formins

In contrast to the Arp2/3 complex, formins are a class of proteins that initiate the nucleation of unbranched actin filaments in a cell (Pollard, 2007; Wallar, 2003). Formins are homodimers in which each monomer has a formin homology 1 (FH1) domain which is made up of proline rich motifs that bind the actin binding protein (ABP)/ profilin complexed with actin as well as a formin homology 2 (FH2) domain that binds actin (Breitsprecher, 2013; Pollard 2007, Kovar, 2004). Nucleation of an actin filament by

formins involves the two FH2 domains forming a dimer which binds to two or three actin monomers and promotes the formation of a nucleus (Pollard, 2007; Goley, 2006).

Spires

Lastly, spires are part of the most recently discovered group of actin nucleators that contain a number of Wiskott-Aldrich syndrome protein homology 2 (WH2) domains which bind actin (Ito, 2011; Quinlan, 2005; Chereau, 2005). Spire contains four WH2 domains which each bind a G-actin molecule and the actin monomers are then arranged to form a tetrameric nucleus to stimulate the polymerization of an actin filament (Quinlan, 2005).

In conclusion, Arp2/3 complex, spire and formins are all actin nucleators that act in different ways to promote nucleation and overcome this rate limiting step in the generation of an actin filament.

Actin turnover/severing proteins

In order for important biological processes to take place in a cell the actin cytoskeleton must undergo coordinated cycles of assembly and disassembly to carry out these events. For example, during cell migration, actin monomers polymerize at the cell's leading edge forming membrane protrusions that drive the cell in a specific direction as well as depolymerize so that G-actin monomers are made available for another round of polymerization (Bailly, 2007). Thus, there are two major proteins that are involved in severing actin filaments which generate smaller fragments that can be used for polymerization or depolymerization and they are called gelsolin and cofilin (Ono, 2007).

Gelsolin

Gelsolin was first isolated from macrophages and was later found to sever actin filaments in the presence of calcium (Yin, 1979; Yin, 1981). Gelsolin along with seven other proteins in mammals is a member of the gelsolin superfamily of proteins which also include CapG, adseverin, flightless I, advillin, villin, villin-like protein and supervillin (Nag, 2013). Members in this family all contain a certain number of conserved gelsolin domains where gelsolin has six named G1-G6 (Nag, 2013). These domains are important in gelsolin carrying out its cellular functions in which G1 binds to G-actin monomers independently of calcium, G2 and G3 bind to F-actin independently of calcium, and G4, G5, and G6 bind to actin monomers in a calcium dependent manner (Ono, 2007; Bryan, 1988; Kwiatkowski, 1985; Way, 1990; Way, 1992b). Moreover, when calcium binds to gelsolin it causes a change in the spatial orientation of the gelsolin domains which allows each domain to bind actin (Nag, 2013). Gelsolin initially binds to a filament with G2G3 and is thought to introduce a change in the twist of the filament which then weakens longitudinal contacts between actin monomers causing the filament to fragment (Nag, 2013; McGough, 1998). Furthermore, G1, G4, G5 and G6 on gelsolin are able to cap severed filament which prevent their polymerization as well as sequester actin monomers (Nag, 2013).

G-actin monomer binding proteins (CAP1)

CAP (adenylyl **c**yclase-**a**ssociated **p**rotein) is a protein that binds G-actin monomers and was originally identified in *Saccharomyces cerevisiae* as SRV2, a gene that is involved in the activation of adenylate cyclase (Hubberstey, 2002; Fedor-Chaiken, 1990). Overall, it was found that mutations made in SRV2 reduced the activation of adenylate cyclase by

RAS stimulation which in turn lowered cAMP (cyclic adenosine monophosphate) levels (Fedor-Chaiken, 1990). It is now known that CAPs are conserved among eukaryotes and they exhibit a variety of different actin regulatory functions although their role in Ras signalling is unique to yeast (Zhang, 2013; Hubberstey, 2002). Mammals have two CAP isoforms called CAP1 which is expressed in all tissues and CAP2 which is expressed in the brain, heart, skeletal muscle and skin (Peché, 2007). Moreover, all CAP homologues contain an N-terminal domain that is followed by a proline rich middle domain, Wiskott-Aldrich Syndrome Protein (WASP) homology 2 (WH2) domain, and a C-terminal end domain (Peché, 2013). In yeast the N-terminal α -helical domain binds to adenylyl cyclase in the Ras/cAMP pathway as well as cofilin (Moriyama, 2002; Makkonen, 2013). Similarly, the N-terminal α -helical domain of CAP1 in mammals has been shown to play an important role in interacting with the cofilin-actin complex and increasing actin turnover rates (Moriyama, 2002). Cofilin is a protein that is involved in severing actin filaments and making smaller fragments available for depolymerization or polymerization (Sidani, 2007). CAP1 functions to increase the actin filament turnover rates with cofilin by enhancing depolymerization at the pointed end of a filament (Moriyama, 2002). Interestingly, a recent study knocked down CAP1 in Hela cells which caused a decrease in phosphorylated cofilin as well as an increase in filamentous actin and enhanced lamellipodia, which increased cell migration and invasion in these cells (Zhang, 2013). In contrast, other studies have shown that eliminating CAP1 caused a decrease in cell migration (Zhu, 2013). For instance, CAP1 is important in the differentiation and migration of Schwann cells where its levels are increased after sciatic nerve injury (Zhu, 2013). CAP1 was shown to interact with actin in these cells and when it was knocked down this caused a decrease in cell migration (Zhu, 2013). Furthermore, studies have

shown that both CAP1 and AIP1 (actin interacting protein-1) interact with the cofilin-actin complex (Moriyama, 2002; Balcer, 2003). AIP1 is the yeast ortholog of WDR1 and is known to enhance the actin filament depolymerizing activity of cofilin by enhancing the severing activity of cofilin and or by capping the barbed ends of severed filaments which prevents them from reannealing and polymerizing (Rodal, 1999; Balcer, 2003). In addition, a yeast two hybrid experiment revealed protein interactions between cofilin, actin, CAP, AIP1 and another protein, profilin (Drees, 2001). In yeast, CAP binds profilin through a proline rich domain whereas mammalian CAP1 contains two separate proline rich profilin binding sites (Makkonen, 2013; Bertling, 2007). Profilin is a small protein that binds G-actin monomers and is able to both inhibit and promote actin filament polymerization depending on its concentration in the cell relative to the amounts of monomeric actin and free barbed ends that are available for polymerization (Ding, 2012; Sohn, 1994; Yarmola, 2009). For instance, when there are capping proteins present to block the barbed ends of filaments from elongation, profilin binds to and sequesters G-actin which prevents it from being incorporated into a new filament (Ding, 2012). In contrast, if there are free barbed ends as well as monomeric actin available, profilin promotes actin filament elongation (Ding, 2012). Profilin is able to promote F-actin polymerization by forming a complex with actin and binding to the barbed end of a filament where actin is deposited before dissociating from the complex (Ding, 2012; Nyman, 2002). Also, profilin stimulates the exchange of ADP to ATP bound G-actin which is then ready for polymerization (Mockrin, 1980; Yarmola, 2009; Ding, 2012). Furthermore, the WH2 domain of CAP1 can bind with higher affinity to ATP G-actin than in yeast where it can then be added to the barbed end of a growing filament (Makkonen, 2013). Moreover, like in yeast, CAP1 also binds ADP G-actin monomers

through its β -sheet C-terminal domain and increases polymerization at the barbed end of a filament as well as promotes the nucleotide exchange of ADP to ATP on actin monomers making them available for polymerization (Moriyama, 2002; Hubberstey, 1996; Makkonen, 2013). Importantly, the promotion of nucleotide exchange on G-actin through the C-terminal domain in CAPs is highly conserved in a variety of organisms ranging from mammals to yeast and even apicomplexan parasites (Makkonen, 2013). Furthermore, although less is known about the functional role of CAP2 in cells, a recent study revealed that when CAP2 is inactivated in mice there is a disruption in the organization of sarcomeres and the mice experience cardiac defects (Peché, 2013). CAP2 resides in the M-line of sarcomeres where through its WH2 domain it is able to sever F-actin and thus control filament length, as well as sequesters G-actin monomers for further rounds of polymerization (Peché, 2013). Moreover, both CAP1 and CAP2 have been implicated in their involvement in cancer cell migration where their up regulation may contribute to the invasive properties observed in these cells such as enhanced cell migration (Effendi, 2013; Tan, 2013).

Cofilin

Cofilin is a 19kDa conserved eukaryotic protein and is a member of the actin-depolymerizing factor (ADF)/cofilin (AC) family of proteins which is made up of multiple isoforms depending on the organism where it is found. For instance, mammals have three ADF/cofilin isoforms which include ADF also called destrin and cofilin-1 which are both found in non-muscle cells, as well as cofilin-2 which is found in muscle cells (Samstag, 2013; Ono, 2007; Ono, 2003; Bamburg, 1999). Moreover, cofilin is able to bind to both G-actin monomers and F-actin filaments in which it contains a G-actin

binding site as well as a G/F actin binding site (Ono, 2003). Studies have demonstrated that cofilin functions in both severing and depolymerizing the actin filaments which are two independent events (Ono, 2007). For example, one study introduced point mutations in porcine cofilin which decreased its actin filament depolymerizing activity but had little effect on the ability of cofilin to sever actin filaments suggesting that these are two separate events (Moriyama, 1999). Firstly, cofilin functions to actively sever the actin filaments, which increases actin filament turnover rates by making free barbed ends available for polymerization or depolymerization (Sidani, 2007). Moreover, a proposed mechanism for cofilin's actin filament severing activity involves it binding to F-actin and causing a twist in the filament which would then weaken the bonds between two adjacent actin monomers and therefore break the filament (McGough, 1997; Ono, 2007). Furthermore, cofilin also increases actin filament turnover rates by depolymerizing actin monomers from the pointed end of a filament (Ono, 2007; Yamashiro, 2005). Interestingly, different cofilin isoforms have been shown to vary in the intensity of their functions depending on their location in a cell (Ono, 2013). For instance, *Caenorhabditis elegans* has two cofilin isoforms which include UNC-60A which is found in the myoepithelial sheath and UNC-60B which is found in the body wall muscle (Ono, 2013, Ono, 2008, Ono, 2003). UNC-60A exhibits a weak ability to sever F-actin but a strong ability to sequester actin monomers. In contrast, UNC-60B shows a strong activity in severing actin filaments whereas its ability to sequester G-actin is negligible (McKim, 1994; Ono, 1998; Yamashiro, 2005; Ono, 2013). Furthermore, cofilin has been shown to play an important role in cell migration where its suppression causes a build-up of filamentous actin and an impairment cell migration (Kato, 2008; Ono, 2007). For example, cofilin is crucial for the directed chemotactic cell migration of T-cells which is

required for them to locate antigen-presenting cells or specific targets and a knockdown in cofilin causes a dramatic decrease in directed cell motility (Samstag, 2013; Klemke, 2010). Likewise, cell migration is also reduced when the signalling kinase MEK is inhibited which normally functions to inhibit LIM-kinase, a protein involved in phosphorylating and inhibiting cofilin (Samstag, 2013; Klemke, 2010). In addition, studies have found that the activity of cofilin contributes to cancer cell invasion and migration (Huang, 2013; Wang, 2010). For instance, studies have found when a serine/threonine kinase called Aurora-A is overexpressed in breast cancer tissue, there is an increase in Slingshot-1, a protein that dephosphorylates and activates cofilin and thus causes an increase in cell migration (Wang, 2010). Moreover, cofilin's activity is regulated in a variety of ways such as changes in pH in which cofilin exhibits stronger actin depolymerizing and severing activities at higher more basic pH levels than at lower acidic pH levels (Ono, 2007; Chen, 2004; Hawkins, 1993). Also, multiple proteins exist in the cell which can alter cofilin's ability to sever and depolymerize actin filaments through phosphorylation and dephosphorylation (Yamaguchi, 2007). For instance, phosphorylation on the serine 3 residue of cofilin by protein kinases such as Lin11, Isl-1 and Mec-3 (LIM)-kinase 1 (LIMK1), LIM-kinase 2 (LIMK2), Testicular protein kinase 1 (TESK1) and Testicular protein kinase 2 (TESK2) make it inactive by preventing it from binding to F-actin and therefore this abolishes its severing and depolymerizing activity (Yamaguchi, 2007; Okano, 1995; Yang, 1998; Toshima, 2001a; Toshima, 2001b; Mizuno, 1994). In contrast to these protein kinases, certain protein phosphatases such as slingshot and chronophin are able to remove a phosphate group on serine 3 of cofilin and make it active (Yamaguchi, 2007; Niwa, 2002; Gohla, 2005). Furthermore, recent studies in breast cancer cells revealed that cofilin's activity can be regulated through protein

glycosylation by *O*-GlcNAc transferase (OGT), a protein that catalyzes the addition of an *O*-linked N-acetylglucosamine (*O*-GlcNAc) to serine 108 on cofilin which is required for its localization in invadopodia. Overall, it was found that a mutation made on serine 108 of cofilin that rendered it unable to become *O*-GlcNacylated also prevented cofilin's localization in the invadopodia leading to impaired breast cancer cell invasion (Huang, 2013). Moreover, cofilin is regulated by a number of proteins that bind to cofilin and inhibit its actin filament severing activity such as phosphatidylinositol-4,5-phosphate (PIP2) and cortactin (Huang, 2013; van Rheenen, 2007; Oser, 2009). Important for this thesis, cofilin has also been shown to be regulated by Actin interacting protein 1 (Aip1p) which can enhance cofilin's actin filament severing activity (Ono, 2003).

Actin Interacting Protein 1 (AIP1) in lower eukaryotes

Actin interacting protein 1 (Aip1p) was originally discovered in the yeast *S.cerevisiae* by introducing thirty five different actin mutants as Gal4p fusion proteins into a yeast two-hybrid system. Overall, it was found that a subset of these mutations disrupted the interaction between actin and a new protein they called Aip1p on subdomains three and four of actin (Amberg, 1995). A few years later Rodal and colleagues used a two-hybrid analysis to show that Aip1p also interacts with cofilin (Cof1p) in yeast. Interestingly, they found that there was an overlap between actin interacting with Aip1p and cofilin in which three of the six mutations made in actin that disrupted its interaction with Aip1p also disrupted cofilin's interaction with actin on subdomain three (Rodal, 1999). Also, it was found that Aip1p is required for the proper localization of cofilin in the cortical actin patches which are highly dynamic structures involved in endocytosis where it colocalizes with cofilin (Rodal, 1999; Okada, 2006).

Furthermore, in 2003 the crystal structure of Aip1p was determined in yeast which revealed the location of some conserved residues that may be important in the interaction between Aip1p with actin or cofilin (Voegtli, 2003). Aip1p is 67kDa and is a member of the WD-repeat protein family (Amberg, 1995; Voegtli, 2003). Proteins in this family all contain WD repeats which are stretches of about 44-60 amino acids long that contain tryptophan aspartate at their carboxyl ends and are thought to play an important role in mediating protein-protein interactions (Luo, 2013; Smith, 1999). WD repeat proteins take on a β -propeller fold structure which is made up of multiple blades, each blade being a four-stranded antiparallel β sheet (Smith, 1999; Voegtli, 2003). Aip1p contains 10 WD repeats and is predicted to be clam-shell shaped with two β -propellers each consisting of seven blades. Furthermore, conserved residues exist between the two β -propellers which could be important in the interactions between Aip1p and its protein substrates such as cofilin and actin (Voegtli, 2003; Ono, 2001). Furthermore, the functional role of Aip1p in yeast is to promote cofilin's activity in disassembling actin filaments (Rodal, 1999). Aip1p carries out its function in yeast by enhancing the severing activity of cofilin and or by capping the barbed ends of the severed filaments which prevents them from reannealing and polymerizing (Michelot, 2013; Balcer, 2003; Okada, 2002; Rodal, 1999). Interestingly, a recent study revealed that Aip1p functions with capping protein (CP) to maintain high concentrations of actin monomers that are ready for another round of productive polymerization in both yeast and mammalian cells (Michelot, 2013). Aip1 and CP were shown to have overlapping functions in maintaining a high concentration of actin monomer pools while at the same time preventing the unproductive elongation of older ADP rich barbed ends (Michelot, 2013). In both yeast and mammalian PtK1 cells, Aip1 localizes in areas where there are ADP rich filaments and blocks CP from binding to

them which in turn forces CP to localize to areas that contain ATP rich F-actin. However, when Aip1 is depleted in these cells, CP is free to localize in areas that contain ADP filamentous actin (Michelot, 2013). Overall, the barbed ends of older filaments are capped by Aip1p which stops their elongation, a process that would most likely occur if CP had to diffuse over to newly severed filaments (Michelot, 2013). Importantly, Aip1p is not essential for survival in yeast but is synthetic lethal when it is depleted in combination with a cofilin mutant (Iida, 1999). In conclusion, Aip1 is found in a variety of different organisms including *Saccharomyces cerevisiae*, *Caenorhabditis elegans* (*C.elegans*), *Xenopus*, *Dictyostelium discoideum*, *Arabidopsis thaliana*, *Drosophila melanogaster* and mammals (Ono, 2003).

D.discoideum

DAIP1, the *D.discoideum* homologue of AIP1, plays an important role in endocytosis, cytokinesis and migration through its interaction with cofilin in promoting rapid actin turnover rates (Choi, 2010; Konzok, 1999). DAIP1 null mutants exhibit a reduction in nutrient uptake during endocytosis, reduced cell motility, and defects in cytokinesis (Konzok, 1999). Furthermore, during chemotactic migration in *D.discoideum*, an increase in intracellular pH causes assembly of actin filaments at the cell's leading edge which propels the cells towards its target (Patel, 2005; Choi, 2010). Cells that do not have NHE1, an Na⁺-H⁺ exchanger that is required for regulating the pH in cells, have a decrease in filamentous actin as well as impairment in chemotactic migration when stimulated by a chemokine (Choi, 2010). Interestingly, DAIP1 has been shown to inhibit the impairment in chemotactic migration as well as cause an increase in F-actin assembly

in NHE1deficient cells and is thought to do so through increasing the activity of cofilin which is low in these cells relative to controls (Choi, 2010).

C.elegans

C.elegans has two Aip1 orthologs called UNC-78 which has 40% sequence identity with WDR1 and AIP1-like gene-1 (AIPL-1) which has 41% amino acid sequence identity with WDR1. Moreover, both UNC-78 and AIPL-1 are essential for embryonic development and reproduction (Ono, 2011; Ono, 2013). The amino acid sequence of AIPL-1 is 66% identical to that of UNC-78 and both proteins interact with UNC-60B (muscle cofilin) in the body wall muscle during embryonic development and UNC-60A (non-muscle cofilin) in the somatic gonad during reproduction, where they act to enhance their actin filament severing activity (Ono, 2011; Ono, 2013). Firstly, when UNC-78 is knocked down in *C.elegans* there is a disorganization of actin filaments in the body wall muscle whereas this phenotype is not observed when AIPL-1 is depleted (Ono, 2001; Ono, 2011). However, when both Aip1 isoforms are knocked down this causes a more severe disorganization of F-actin in the body wall muscle and ultimately leads to death of embryos which suggests that UNC-78 and AIPL-1 have overlapping functions in the proper development of early embryos in *C.elegans* (Ono, 2011). Interestingly, similar to the requirement for Aip1p in the localization of cofilin in actin patches in yeast, UNC-78 and AIPL-1 are important in the localization of UNC-60B in the cytoplasm of the body wall muscle in *C.elegans* embryos (Rodal, 1999; Ono, 2011). Depletion of UNC-78 and AIPL-1 caused an enhanced mislocalization of UNC-60B to aggregates compared to a depletion of UNC-78 alone which had a milder effect or AIPL-1 alone which had no effect (Ono, 2011). Similarly, UNC-78 and AIPL-1 have redundant roles in reproduction

in *C.elegans* and when they are both knocked down the actin cytoskeleton is disrupted in the somatic gonad which interferes with the contractile activity in the myoepithelial sheath that is required to direct the oocyte to the spermatheca and leads to sterility (Ono, 2013).

Drosophila

The *Drosophila* homolog of Aip1p is encoded by the flare (flr) gene (Ren, 2007). In contrast to yeast where cofilin but not Aip1p is essential for survival, both are essential for survival in *Drosophila* (Ren, 2007). Furthermore, mutations in both flr and twinstar (tsr) which is *Drosophila* cofilin, cause abnormal morphologies in hair cells of the *Drosophila* wing which are likely a result of defects in actin filament turnover rates in these cells (Ren, 2007). Furthermore, flr and tsr have also been shown to play an important role in eye development in *Drosophila* and a mutant loss of function flr causes an increase in actin filaments in cluster cells of the adherens junctions (Chu, 2012). Overall, this prevents remodelling of adherens junctions to occur which is necessary for cells to be arranged appropriately in the cell sheets during epithelial morphogenesis and ultimately leads to eye defects in *Drosophila* (Chu, 2012).

Plants

In plants, high actin turnover rates are required for tip growth which is the polarized cell expansion in tissues such as root hairs which are important for the acquisition of nutrients as well as pollen tubes which are involved in plant reproduction (Augustine, 2011; Hepler, 2001; Menand, 2007). AIP1 and cofilin have been shown to play an important role in the dynamic reorganization of the actin cytoskeleton in plants

which include *Physcomitrella patens* (*P. patens*), *Arabidopsis thaliana* (*A.thaliana*) and *Oryza sativa* (*O.sativa*) (Augustine, 2011; Shi, 2013; Ketelaar, 2007). Firstly, the moss *P.patens* has a single AIP1 and a single ADF gene which work together to promote actin filament turnover of actin filaments (Augustine, 2011). When AIP1 was knocked down in cells this caused an increase in actin filament bundles in tip growing cells which resulted in plants with smaller cells and reduced growth although they were viable (Augustine, 2011). However, ADF is essential for plant survival and its knockdown inhibited growth of cells in which they did not survive (Augustine, 2011; Augustine, 2008). Similarly in *O.sativa* OsAIP1 promotes ADF's actin filament severing and depolymerizing activity and when it was overexpressed actin filament assembly was inhibited whereas when it was underexpressed there was an increase in actin filament assembly observed in root hairs (Shi, 2013). Furthermore, *A. thaliana* has two AIP1 genes named AIP1-1 and AIP1-2 which are important in plant development (Allwood, 2002; Ketelaar, 2007). AIP1 is essential for cell viability in *A.thaliana* and when it was inhibited in these plants there was an accumulation of F-actin in root, shoot and leave cells which reduced their growth and even caused plant death in the most extreme cases (Ketelaar, 2004). In contrast, when AIP1 was overexpressed there were higher actin turnover rates as well as a decrease in F-actin in root hairs in which they were shorter and swollen compared to normal cells (Ketelaar, 2007).

Xenopus laevis

XAIP1 is the *Xenopus* homolog of AIP1 and has been shown to interact with and promote the activity of XAC (*Xenopus* cofilin) in severing actin filaments by capping the barbed ends and thus preventing the severed filaments from reannealing and polymerizing

(Okada, 1999; Okada, 2002). Moreover, both XAIP1 and XAC are localized in the cortical actin cytoskeleton and the cleavage furrow in frog embryos (Okada, 1999). XAIP1 plays an important role in embryonic development in *Xenopus* in which its overexpression prevents the accumulation of F-actin in the cleavage furrow in blastomeres which is required for cleavage to occur (Okada, 1999; Ono, 2003).

AIP1/WDR1 in higher eukaryotes

WDR1 is the mammalian ortholog of Aip1p and is a 51kDa protein that is 606 amino acids long and contains 9 WD repeats (Haslenc-Hox, 2013). Very little is known about the function of WDR1 except that it is thought to enhance cofilin's ability to sever and depolymerize actin filaments (Kato, 2008; Ono, 2003; Ono, 2007; Izawa, 2013). The WDR1 gene was first identified in chicks where its mRNA levels were upregulated in the auditory epithelium known as the basilar papilla after noise damage. It was found to be highly conserved, having 86% sequence identity as well as 98% similarity in amino acid sequence with human and mouse WDR1 (Adler, 1999). Unlike mammals, birds are able to reverse and even fully recover from hearing loss after noise damage although the molecular mechanisms behind the repair process are unclear (Adler, 1999). In 2002, Oh and colleagues found that WDR1 colocalized with both ADF and actin in the inner ear of chicks and after noise damage WDR1 was upregulated in support cells that surround hair cells in the inner ear (Oh, 2002). Overall, this gave insight into a possible mechanism by which WDR1 may help reverse noise damage by enhancing ADF/cofilin's ability to sever and depolymerize actin filaments and thus contribute to the necessary reorganization of the actin cytoskeleton after acoustic overstimulation (Oh, 2002). Lastly, over a decade later studies revealed that WDR1 is also upregulated in the cochlea of Sprague-Dawley

rats after acoustic overstimulation compared to normal controls (Song, 2013). In conclusion, although the role of WDR1 in noise damaged inner ear cells is not fully understood it may serve to protect against damage of the actin cytoskeleton from noise overstimulation (Song, 2013).

WDR1 has been shown to play an important role in both cell migration and cytokinesis via its role in increasing actin turnover rates with cofilin (Kato, 2008). For instance, when WDR1 was knocked down in Hela cells with small interfering RNA (siRNA) there was a build-up of filamentous actin near the contractile ring in telophase which prevented cytokinesis from occurring properly and lead to the formation of multinucleate cells. Moreover, when WDR1 was knocked down in Jurkat cells, an impairment in directed chemotactic cell migration was observed in the cells, where multiple cell protrusions were formed compared to a single lamellipodium observed in normal cells after stimulation with a chemokine. Overall, both scenarios demonstrate the critical role of WDR1 in enhancing cofilin's ability to sever F-actin filaments which is required for both directed cell migration and cytokinesis to properly take place (Kato, 2008). More recent studies have implicated the importance of WDR1 in cancer cell migration and invasion in both breast cancer and ovarian cancer (Haslene-Hox, 2013; Kang, 2010; Kim, 2011). Firstly, WDR1 is upregulated in the interface zone in breast cancer tissue which is the region existing between tumor and normal tissues as well as the interstitial fluid of ovarian cancer tissues (Haslene-Hox, 2013; Kang, 2010). Overall, it may play an important role in contributing to the invasive properties in these different cancer cell types by generating higher actin turnover rates along with cofilin at the cell's leading edge which would give rise to the characteristic increase in directed cell

migration and invasion unique to cancer cells (Haslène-Hox, 2013; Kang, 2010; Kato, 2008). Interestingly, WDR1 has been shown to localize in the core of podosomes in macrophages (Cervero, 2012). Podosomes are the specialized organelles found in invasive cell types that contain an actin rich core as well as a variety of actin associated proteins (Chhabra, 2007; Cervero, 2012). Therefore, it is not surprising that WDR1 is upregulated in these structures in which it could assist with the dynamic reorganization of the cytoskeleton in the F-actin rich core of the podosome (Cervero, 2012). Furthermore, another study that made different mutations in WDR1 to understand its functional role better in mice found the most severe loss of function alleles lead to death of early embryos whereas less extreme mutations caused thrombocytopenia and autoinflammatory disease (Kile, 2007). Firstly, autoinflammatory disease resulted from a mutation in WDR1 that caused an accumulation of F-actin in neutrophils which ultimately decreased their directional cell migration rates and most likely initiated an inflammatory response. In contrast, macrothrombocytopenia was a result of megakaryocytes not being able to mature properly and undergo the rearrangements of the actin cytoskeleton which are required for proper production of platelets (Kile, 2007). Interestingly, WDR1 has been found to be down regulated in platelets of both smokers and uraemic patients which could give valuable insight into the molecular mechanisms behind the abnormal platelets observed in these individuals which still remains unclear (Plé, 2012; Della Corte, 2012). Furthermore, contrary to the predicted functional role of WDR1 in enhancing cofilin's actin filament severing activity, one study demonstrated that WDR1 may instead inhibit cofilin's activity during mitosis (Fujibuchi, 2005). When WDR1 was knocked down in LAK/T cells this caused cells that were undergoing mitosis to flatten out instead of round up which usually occurs. Moreover, they found that phosphorylated cofilin was able to

still carry out its actin filament severing and depolymerizing activity to a certain degree but in the presence of WDR1 its activity was suppressed which may be necessary for mitosis to take place (Fujibuchi, 2005). Lastly, WDR1 may be an be a good candidate gene in both bipolar disorder and schizophrenia in which there is significant linkage between these disorders to a region on chromosome 4p where WDR1 is located (Le Hellard, 2007; Wang, 2011). However, the molecular mechanisms underlying how WDR1 may contribute to the pathogenesis of bipolar disorder and schizophrenia has yet to be determined and is a good topic for future investigation (Le Hellard, 2007; Wang, 2011).

WDRΔ35

A few years ago the Hubberstey lab discovered a splice variant of WDR1, WDRΔ35, which is ~42kDa and lacks exons 3-5. It is 466 amino acids long, and is missing 3 of the 9 WD repeats found in WDR1 (Tousignant, R. et al., manuscript in preparation). Importantly, WD repeats are known to play an important role in mediating protein-protein interactions and therefore the functional role of WDRΔ35 may be altered relative to WDR1 because it is missing 3 of these WD repeats (Luo, 2013; Smith, 1999). However, currently nothing is known about the function of WDRΔ35 other than expression levels of the two genes are differentially expressed in human tissues and a variety of cancer cell lines (Tousignant, R. et al., unpublished). Also, cofilin overexpression in HEK293 cells caused a significant increase in WDRΔ35 mRNA levels, which suggests a potential linkage between WDRΔ35 and cofilin (Correa, R. et al., manuscript in preparation). One of the major objectives of this study is to determine whether any functional differences may exist when these two proteins are overexpressed

in mammalian cells. Furthermore, are there specific amino residues that may affect the ability of these proteins to impair cell migration.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture

HEK (Human Embryonic Kidney) 293 cells (ATCC) were cultured in High Glucose DMEM (Dulbecco's modified Eagle medium) (Thermo scientific) supplemented with 10% FBS (Fetal Bovine Serum) (Thermo scientific) and 1% Pen Strep (Penicillin Streptomycin) antibiotics (Sigma-Aldrich). For subculturing, culture medium was removed from dish. Next cells were washed with 5ml PBS (Phosphate-buffered saline) (137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄ and 1.8mM KH₂PO₄ with a pH of 7.4). The PBS was discarded and 2ml of 0.25% Trypsin-EDTA (Thermo Scientific) was added to dish. The dish was incubated for 2 minutes at 37°C and 5% CO₂. Next 8ml of culture medium was added and the cell suspension was spun at 572 x g for 5 minutes at 25°C. The media was removed and the cell pellet resuspended in 10ml of new media. The appropriate amount of the cell suspension was added to new culture dishes filled with 10ml of media. The cultures were incubated at 37°C with 5% CO₂. The cells were subcultured when they were 95-100% confluent.

Transfections

The following procedure (Invitrogen) was used to transfect DNA into mammalian cells in a 35mm tissue culture dish (Sarstedt): One day before transfection cells were plated at $2-4 \times 10^5$ cells in 2ml of culture medium so that cells were 80% confluent at the time of transfection. Then for each transfection sample the complexes were prepared as follows:

- a. 4µg of DNA was diluted in 250µl of High Glucose DMEM culture medium without serum and antibiotics.
- b. 10µl of LipofectamineTM2000 (Invitrogen) was diluted in 250µl of High Glucose DMEM culture medium without serum and antibiotics and incubated for 5 minutes at room temperature.
- c. After the 5 minute incubation, the diluted DNA and the diluted LipofectamineTM2000 (total volume = 500µl) were combined and incubated for 20 minutes at room temperature.

Next, the 500µl complexes were added to each culture dish containing cells and medium. The cells were incubated at 37°C with 5% CO₂ for 24 hours before testing for transgene expression and the media was changed after 4 hours to High Glucose DMEM supplemented with 10% FBS and no antibiotics.

Site-directed mutagenesis

Plasmids coding for GFP (green fluorescent protein)-tagged WDR1 or WDRΔ35 were constructed by subcloning PCR-amplified mouse WDR1 and human WDRΔ35 cDNA into pEGFP-C1 (Clontech) expression vectors by previous students in the lab (manuscript under preparation). Appendix A lists all the primers used for introduction of mutations in conserved residues of WDR1 and WDRΔ35 (Sigma-Aldrich). The GENEART[®] Site-Directed Mutagenesis System was used for in vitro site-directed mutagenesis of plasmids (Life technologies). For the methylation and mutagenesis reactions the following reaction mixture was prepared: 1X AccuPrimeTM Pfx Reaction mix (dNTPs, MgSO₄, thermostable Accuprime proteins), 1X Enhancer, 0.3µM forward primer, 0.3µM reverse primer, 20ng

of plasmid DNA, 4 units of DNA Methylase, 1X SAM, 1 unit of AccuPrime™ *Pfx* in PCR water. The polymerase chain reaction (PCR) was performed using the following parameters: 1 cycle at 37°C for 20 minutes, 1 cycle at 94°C for 2 minutes, 18 cycles at 94°C for 20 seconds, 18 cycles at 57°C for 30 seconds, 18 cycles at 68°C for 3 minutes, 1 cycle at 68°C for 5 minutes, and 1 cycle at 4°C until finished. After the reaction, 5µl of the product was analyzed on a 0.8% agarose gel. Next, for the recombination reaction the following reaction mixture was prepared: 1X Reaction Buffer (4µl of 5X stock), 10µl PCR water, 4µl PCR sample, and 1X Enzymer mix (2µl of 10X stock). The recombination mixture was mixed well and incubated at room temperature for 10 minutes. Next 1µl of 0.5M EDTA was added to stop the reaction and put on ice. For the transformation reaction a 50µl vial of One Shot® MAX Efficiency® DH5α™-T1^R competent cells was thawed on ice for 5 to 7 minutes before adding 2µl of the recombination reaction directly into the vial of cells and incubating them for 12 minutes completely covered in ice. Next the tube was incubated in a 42°C water bath for 30 seconds and then covered with ice for 2 minutes. The vial was removed from ice and 250µl of SOC medium (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose) was added to the vial. The vial was incubated at 37°C while shaking at 225 rpm for 1 hour. Next 10µl of the transformation reaction was diluted with 90µl of SOC medium (total volume of 100µl) and plated on a LB agar plate (10g Tryptone, 5g yeast extract, 10g NaCl and 15g Agar in 1L distilled water) with kanamycin antibiotics (50µg/ml). Plate was incubated for 16-20 hours at 37°C. A pUC19WHITE control plasmid that contains a mutation in the *LacZα* gene was provided as a positive control along with the control primer forward- 5'CGTCGTGACTGGGAAAACCCTGGCGTTACC3' and the control primer reverse-

5'GGTAACGCCAGGGTTTTCCCAGTCACGACG3'. It produced white colonies when plated on a LB agar plate (10g Tryptone, 5g yeast extract, 10g NaCl and 15g Agar in 1L distilled water) with ampicillin (Bio Basic Inc) antibiotics (100µg/ml) and 50µg/ml of X-gal (Fisher Scientific).

Plasmid DNA Isolation (Mini-prep)

A minimum of 3 colonies were picked from each plate from the transformation reaction of the site-directed mutagenesis kit. The GenElute™ Plasmid Miniprep Kit (Sigma) was used to isolate plasmid DNA as per manufacturer's instructions. DNA from each colony was sent out for sequencing (Robarts Research Institute- Western University) and samples were prepared with 1µg DNA and 5µM of sequencing primer. See Appendix A for the sequencing primers that were used for WDR1 and WDRΔ35 mutants. Next, the sequences were aligned on Clustal with the original sequence to confirm that the mutation was made.

Molecular biology procedures

Bacterial transformations, plasmid maxi-preps and gel electrophoresis of plasmids used for transfection were carried out as per manufacturers' instructions.

Protein Extraction and Co-immunoprecipitation (Co-IP)

For each Co-IP experiment: one day before transfection cells were plated at 2×10^6 cells in 10ml of culture medium so that cells were 80% confluent at the time of transfection. Then for each transfection sample the complexes were prepared as follows: a. 8µg of DNA was diluted in 250µl of High Glucose DMEM culture medium without serum and

antibiotics. b. 20µl of LipofectamineTM2000 (Invitrogen) was diluted in 250µl of High Glucose DMEM culture medium without serum and antibiotics and incubated for 5 minutes at room temperature. c. After the 5 minute incubation, the diluted DNA and the diluted LipofectamineTM2000 (total volume = 500µl) were combined and incubated for 20 minutes at room temperature. Next, the 500µl complexes were added to each culture dish containing cells and medium. The cells were incubated at 37°C with 5% CO₂ for 24 hours before testing for transgene expression and the media was changed after 4 hours to High Glucose DMEM supplemented with 10% FBS and no antibiotics. The following procedure (chromotek) was used for a 10cm tissue culture dish with cells that were 95-100% confluent: Culture medium was removed from dish. Next cells were washed with 1ml PBS (Phosphate-buffered saline) (137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄ and 1.8mM KH₂PO₄ with a pH of 7.4) and scraped off from the petri dish. They were then transferred to a pre-cooled tube and spun at 572 x g for 5 minutes at 4°C. PBS was removed from the pellet and it was resuspended and washed two more times with PBS. Next the pellet was resuspended in 200µl of lysis buffer (10mM Tris/Cl pH 7.5, 150mM NaCl, 0.5mM EDTA, 0.5% NP40, 1 Protease inhibitor cocktail tablet (Roche Diagnostics)). The tube was placed on ice and pipetted up and down every 15 minutes for 30 minutes. The cell lysate was spun at 14 000 x g for 10 minutes at 4°C. The supernatant was transferred to a pre-cooled tube and the volume was adjusted to 500µl with dilution buffer (10mM Tris/Cl pH 7.5, 150mM NaCl, 0.5mM EDTA, 1 Protease inhibitor cocktail tablet). Next for immunoblot analysis 50µl of the cell lysate was diluted with 50µl of 2X SDS-sample buffer (20% Glycerol, 120mM Tris/HCl pH 6.8, 4% SDS, 0.02% bromophenol blue, 5% beta-mercaptoethanol) and 30µl of sample was run on a 10% SDS polyacrylamide gel (Resolving: 1.5M Tris/Cl pH 8.8, 40% Acrylamide (Sigma-Aldrich),

10% SDS, 10% ammonium persulfate (APS) (Sigma-Aldrich), 10µl Temed (Bio Basic Inc); Stacking gel: 0.5M Tris/Cl pH 6.8, 40% Acrylamide, 10% SDS, 10% APS, 10µl Temed). For a co-immunoprecipitation experiment GFP-Trap®_A beads (Chromotek) were equilibrated by resuspending 20µl of bead slurry in 500µl of dilution buffer. The beads were spun down at 2500 x g for 2 minutes at 4°C, the supernatant was removed, and the beads were washed 2 more times with dilution buffer. Next the cell lysate was added to the equilibrated GFP-Trap®_A beads and incubated while shaking for 1 hour at 4°C. After 1 hour, the tube was spun at 2500 x g for 2 minutes at 4°C and then 50µl of supernatant was diluted with 50µl of 2X SDS-sample buffer for western blot analysis. Next the beads were washed 3 times with 500µl of dilution buffer and then resuspended in 100µl of 2X SDS-sample buffer. The beads were then boiled for 10 minutes at 95°C to dissociate the immunocomplexes from the beads, and then spun at 2500 x g for 2 minutes at 4°C. Lastly, 30µl of the supernatant was run on a 10% SDS polyacrylamide gel.

G-actin/F-actin In Vivo Assay Kit

To measure the ration of F/G actin in the cell, an assay was used (Cytoskeleton Inc.) for a 35mm tissue culture dish with cells that were 95-100% confluent: One day before transfection cells were plated at 4×10^5 cells in 2ml of culture medium so that cells were 80% confluent at the time of transfection. Next 24 hours after transfection culture medium was removed from dish and transferred to a tube where it was spun at 572 x g for 5 minutes at 25°C. The supernatant was removed and the pellet was washed with PBS twice where it was spun at 572 x g for 5 minutes at 25°C. 100µl of LAS2 buffer (1ml LAS01 buffer (Lysis and F-actin Stabilization Buffer), 10µl BSA04 (100mM ATP stock), 10µl PIC02 (100X protease inhibitor cocktail stock) was added to dish after being pre-

warmed to 37°C for 30 minutes prior to beginning the assay. Note: an exception is cells that were treated with 0.1µM of the actin polymerizing drug jasplakinolide (Sigma-Aldrich) 1 hour prior to collecting them. Cells were scraped with a cell scraper and the cell lysate was pipetted into the tube containing the washed pellet that was initially spun down from dish. The sample was then homogenized using a 200µl pipette and pipetting up and down extensively. The cell lysate was then incubated at 37°C for 10 minutes and then centrifuged at 350 x g for 10 minutes at room temperature. The supernatant was transferred to an ultracentrifuge tube and centrifuged at 100 000 x g for 1 hour at 37°C. Next the supernatant was removed from tube (contains G-actin) while being careful not to disturb pellet (contains F-actin). Next, 100µl of F-actin depolymerizing buffer was added to pellet and incubated on ice for 1 hour while pipetting up and down several times every 15 minutes in order for actin filament depolymerization to occur. Lastly, 25µl of 5X SDS sample buffer (30% Glycerol, 250mM Tris/HCl pH 6.8, 10% SDS, 0.02% bromophenol blue, 5% beta-mercaptoethanol) was added to pellet and supernatant samples where 5µl of supernatant and 10µl of pellet sample was run on a 10% SDS polyacrylamide gel. All experiments were done in triplicates.

Western Blot Analysis

Unless specified samples were boiled for 5 minutes at 95°C, vortexed, and then centrifuged at 350 x g for 2 minutes. Samples (30µl unless specified; For F/G actin samples: 5µl of supernatant and 10µl of pellet sample) were then run on a 10% SDS polyacrylamide gel. The proteins were transferred from SDS-PAGE to a nitrocellulose transfer membrane (Fisher Scientific) at 4°C for 1 hour at 100V with Transfer buffer (25mM Tris/Cl, 192mM glycine, 20% methanol). After the transfer, the membrane was

blocked in TBST/5% non-fat milk (10mM Tris/Cl pH 7.4, 150mM NaCl, 0.05% Tween 20 (Fisher Scientific)/5% non-fat milk) for 1 hour at room temperature. For the G-actin/F-actin in vivo assay kit the membrane was blocked in TBST/5% non-fat milk (10mM Tris/Cl pH 7.4, 150mM NaCl, 0.01% Tween 20 (Fisher Scientific)/5% non-fat milk) for 30 minutes at room temperature. Next the membrane was incubated in primary antibody overnight or for 2 hours at room temperature. For the G-actin/F-actin in vivo assay kit the membrane was washed 3 times for 10 minutes in TBST at room temperature before placing it in primary antibody. Primary antibodies that were used in this study include: Anti-actin rabbit polyclonal antibody (Cytoskeleton Inc.) that was diluted at 1:500 in TBST/0.1% non-fat milk. Anti-GFP mouse monoclonal antibody (Santa Cruz) was diluted at 1:1000 in TBST/4% non-fat milk. Anti-actin mouse monoclonal antibody (Cedarlane) was diluted at 1:10000 in TBST/4% non-fat milk. Anti-cofilin rabbit polyclonal antibody (Cytoskeleton Inc.) was diluted at 1:1000 in TBST/4% non-fat milk. Platelet cell extract (cytoskeleton) was loaded (20 μ g) as a positive control for cofilin. Anti-CAP1 rabbit polyclonal antibody (Novus Biologicals) was diluted at 1:1000 in TBST/4% non-fat milk. Next, the membrane was washed 3 times for 5 minutes in TBST at room temperature. An exception is for the G-actin/F-actin in vivo assay kit where the membrane was washed 3 times for 10 minutes in TBST at room temperature. The membrane was incubated with anti-mouse-IGG-HRP (Invitrogen) or anti-rabbit-IGG-HRP (Invitrogen) secondary antibodies depending on the primary antibody that was used for 1 hour at room temperature. The secondary antibody was diluted to 1:10000. Next the membrane was washed 3 times for 5 minutes in TBST at room temperature. An exception is for the G-actin/F-actin in vivo assay kit where the membrane was washed 5 times for 10 minutes in TBST at room temperature. The membrane was then processed for

chemiluminescent detection of proteins using Supersignal West Femto Maximum Sensitivity Substrate (Fisher Scientific).

Wound healing assay and microscopy

HEK293 cells were plated in culture inserts (Ibidi) one day before transfection at $1.1-1.4 \times 10^4$ cells in 70 μ l of culture medium in each well so that cells were 80% confluent at the time of transfection. Cells were transfected with 0.14 μ g of GFP DNA as well as WDR1, WDRR17G, WDRH188Q, WDRG299E, WDRG344E, WDRH531Y, WDRW602STOP, WDR Δ 35, WDR Δ 35H48Q, WDR Δ 35G159E, and WDR Δ 35G204E GFP fusion genes, and 0.7 μ l of lipofectamine per 70 μ l well. For each transfection sample the complexes were prepared as follows: a) 0.14 μ g DNA was diluted in 35 μ l of High Glucose DMEM culture medium without serum and antibiotics b) 0.35 μ l of LipofectamineTM2000 (Invitrogen) was diluted in 35 μ l of High Glucose DMEM culture medium without serum and antibiotics and incubated for 5 minutes at room temperature c) After the 5 minute incubation, the diluted DNA and the diluted LipofectamineTM2000 (total volume = 70 μ l) were combined and incubated for 20 minutes at room temperature. Next, the 70 μ l complexes were added to each well of the culture insert containing cells and medium. The cells were incubated at 37°C with 5% CO₂ for 24 hours before testing for transgene expression and the media was changed after 4 hours to High Glucose DMEM supplemented with 10% FBS and no antibiotics. One day after transfection, the culture insert was removed and 2ml of culture medium was added to the 35mm tissue culture dish. Video microscopy was used to examine the wound healing process. The 35mm tissue culture dish was placed in a live cell chamber at 37°C and 5% CO₂ for 24 hours and images were taken every 10 minutes using the LAS AF6000 software on the LEICA

DM16000 fluorescent microscope to generate live cell time lapse movies. Images were taken in bright field as well as GFP field at 10X magnification. A total of 3 videos were taken for WDR1, 2 videos were taken for WDR Δ 35 and the GFP control, and 1 video was taken for the WDR1 and WDR Δ 35 mutants.

Statistical Analysis

For live cell imaging experiments, the image j manual tracking tool was used to track 20 cells per video and then analyzed using the image j chemotaxis tool to get the average velocity in microns per minute (μ M/min) which was then plotted on a graph using SPSS software. A one-tailed unpaired t-test was used to determine the significance between two groups. A p-value which is less than 0.05 is considered to be statistically significant. A p-value which is less than 0.01 is considered to be very statistically significant. A p-value which is less than 0.0001 is considered to be extremely significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

For the G-actin/F-actin in vivo assay kit bands were quantified using densitometry on Image J software and then plotted on a graph similarly using SPSS software.

CHAPTER 3

RESULTS

Mutational analysis to define active functional domains in WDR1 and WDRΔ35

Previous studies in yeast revealed key residues in Aip1p that are conserved and may be important in its interactions with protein substrates such as actin and cofilin (Voegtli, 2003). However, very little is known about the functional domains that exist in WDR1. Therefore, in order to examine potential active functional domains in WDR1 and WDRΔ35, site-directed mutagenesis was used to introduce point mutations in WDR1 and WDRΔ35 that correspond to highly conserved residues across species (Fig.3.1C, D). The mutations introduced in WDR1 include WDRR17G, WDRH188Q located in WD4 of WDR1, WDRG299E, WDRG344E located in WD6 of WDR1, WDRH531Y located in WD8 of WDR1, and WDRW602STOP located in WD9 of WDR1 (Fig.3.1 A,C). Mutations made in WDRΔ35 include WDRΔ35H48Q located in WD1 of WDRΔ35, WDRΔ35G159E, and WDRΔ35G204E located in WD3 of WDRΔ35 (Fig.3.1 B, D). Importantly, verification that the mutants were stable and cells were expressing the mutant protein was done prior to monitoring the effects of WDR1, WDRΔ35 and the described mutants on cell migration. HEK293 cells were transfected with GFP-tagged WDR1, WDRΔ35 and the various mutants and then lysed and collected after 24 hours. Samples were run on a Western to test expression levels and stability (Fig.3.2, A-E). Fig.3.2 shows western blots with the proper expression of the WDR1 and WDRΔ35 mutants. A band showed up for WDR1 and the WDR1 mutants fused to GFP at 88kDa, and for WDRΔ35 and the WDRΔ35 mutants at 70kDa which confirmed that they were

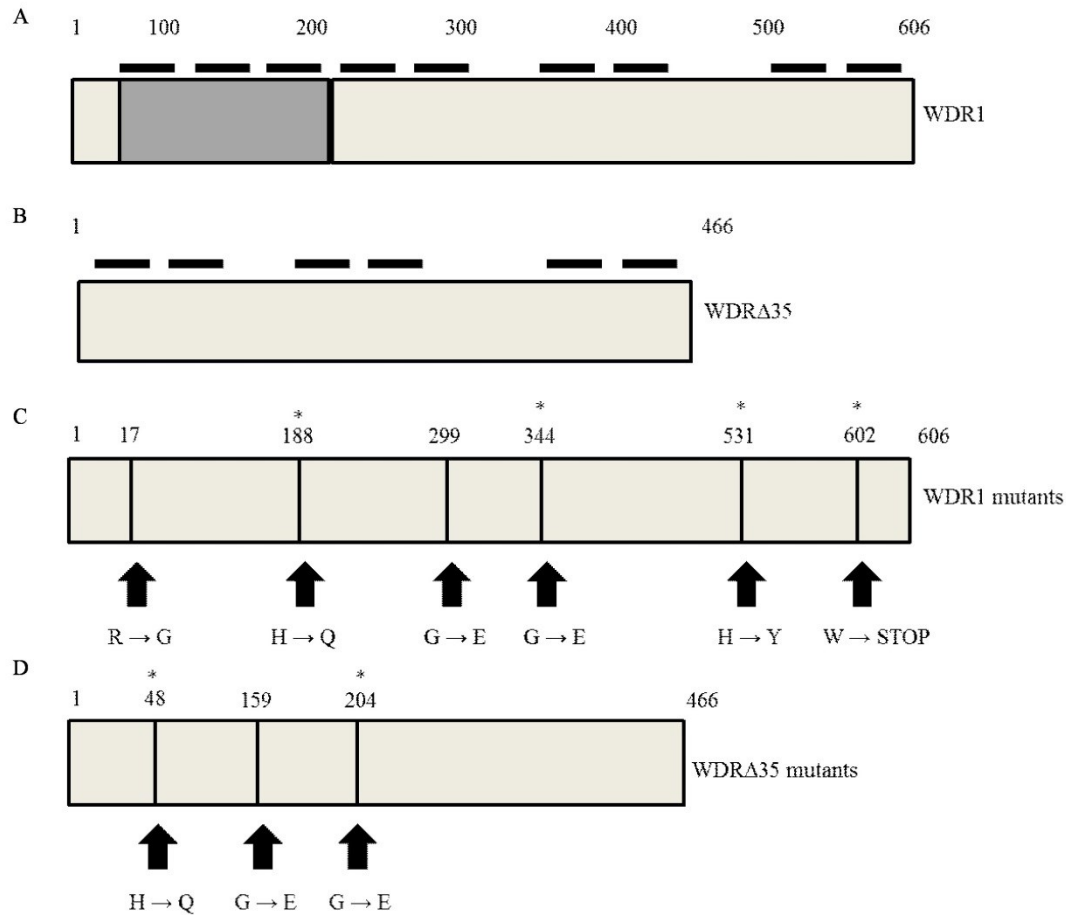


Figure 3.1: Schematic diagram of full length WDR1 and WDRΔ35 and the point mutations made in WDR1 and WDRΔ35. Site-directed mutagenesis was used to introduce point mutations in conserved residues in both WDR1 and WDRΔ35 which may be important in their interactions with protein substrates. A) Full length WDR1. The thick bars above the gene represent the WD repeat regions. The shaded region represents the region missing in WDRΔ35. B) Full length WDRΔ35. The thick bars above the gene represent the WD repeat regions. Notice three are missing from the spliced region of exons 3-5. C) Mutations made in WDR1 include WDRR17G, WDRH188Q, WDRG299E, WDRG344E, WDRH531Y and WDRW602STOP. B) Mutations made in WDRΔ35 include WDRΔ35H48Q, WDRΔ35G159E and WDRΔ35G204E. Asterisks represent mutations that were made in WD repeat regions.

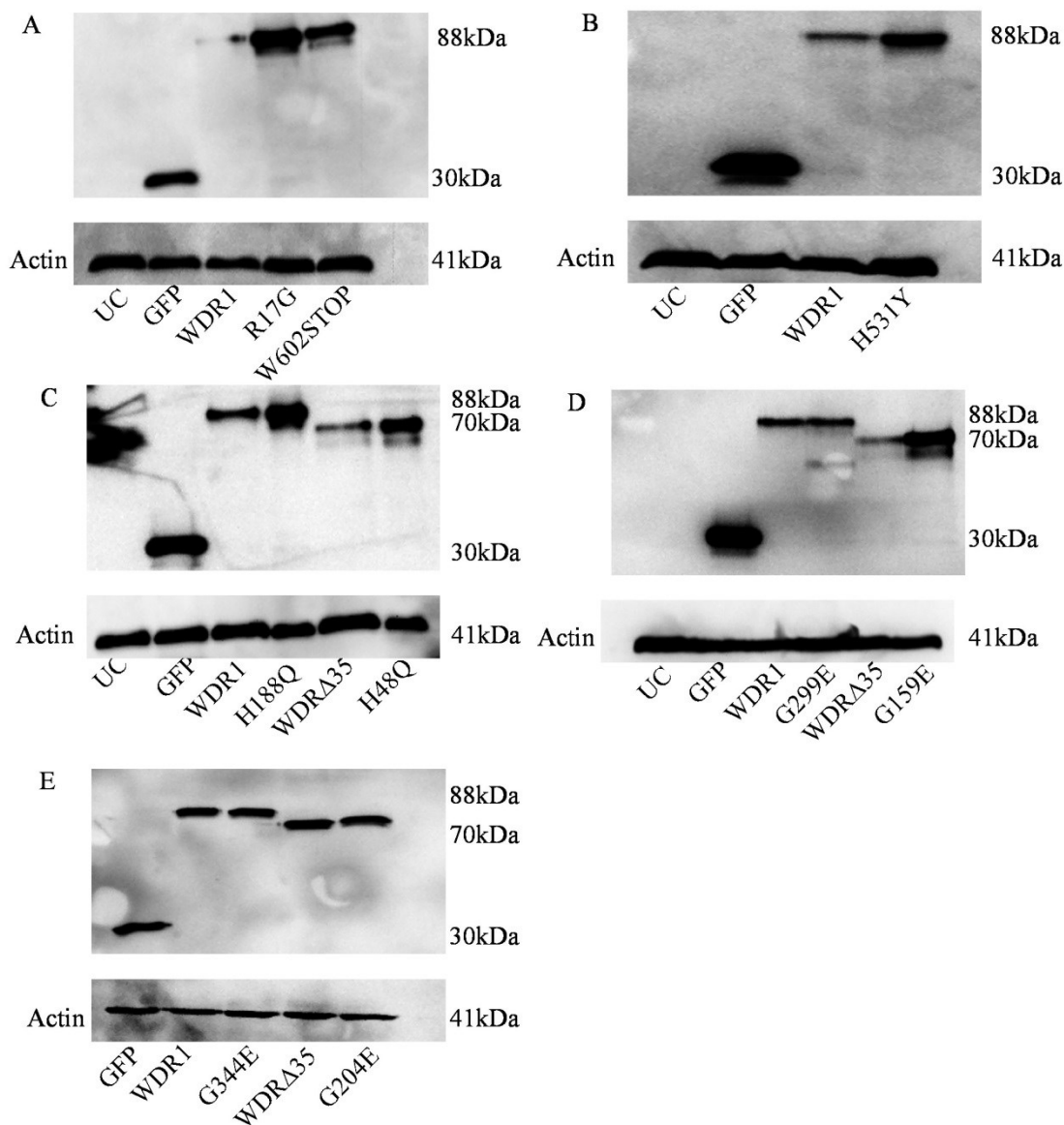


Figure 3.2: Protein expression levels of GFP tagged WDR1 and WDRΔ35 mutants. HEK293 cells were transfected with the various GFP fusion plasmids and then lysed and collected after 24 hours. Samples were run on a Western to test expression levels/stability. UC represents the untransfected control and GFP represent the GFP control. The mutants are shown below the various lanes: (A) WDRR17G and WDRW602STOP, (B) WDRH531Y, (C) WDRH188Q and WDRΔ35H48Q, (D) WDRG299E and WDRΔ35G159E and (E) WDRG344E and WDRΔ35G204E.

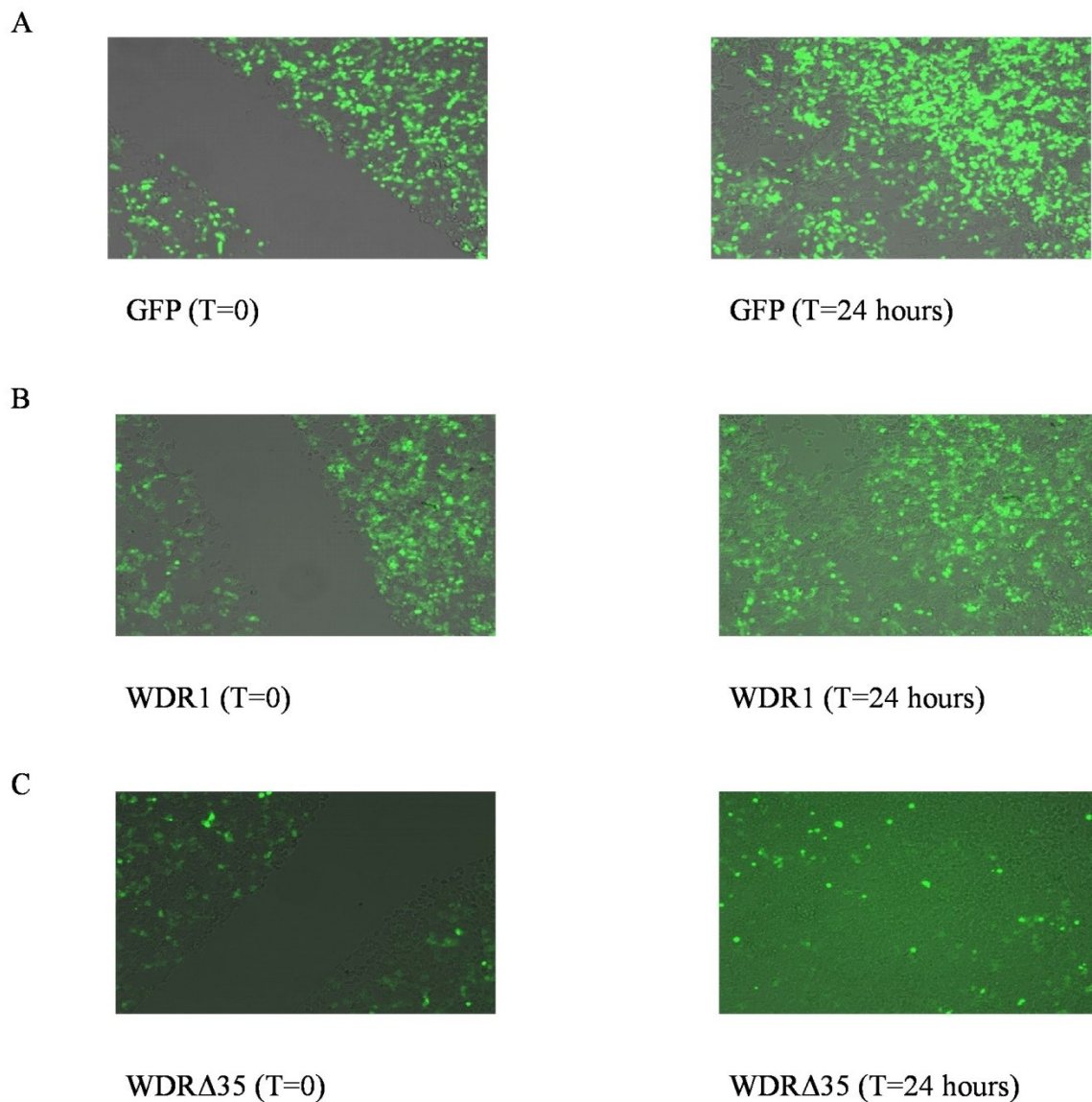


Figure 3.3: Wound healing assay with HEK293 cells overexpressing GFP, WDR1 and WDRΔ35. HEK293 cells were plated in ibidi culture inserts 24 hours prior to transfection with GFP DNA as well as WDR1 and WDRΔ35 GFP fusion genes. Next, 24 hours after transfection the culture insert was removed and video microscopy was used to examine the wound healing process. Bright field and fluorescent images were taken every 10 minutes for 24 hours. Shown are bright field and fluorescent merged images of cells that were transfected with pEGFP (3.3A), pEWDR1 DNA (3.3B) and pEWDRΔ35 DNA (3.3C) at time zero and after 24 hours.

stable and not being degraded in the cell. Moreover, when the cells were examined under a fluorescent microscope they were green which further indicated that the cells were expressing the various mutant proteins fused to GFP (Fig.3.3).

Cell migration rates of WDR1, WDRΔ35 and the described mutants

In order to unveil the effects that WDR1 and WDRΔ35 have on cell migration in mammalian cells HEK293 cells were plated in Ibidi culture inserts 24 hours prior to transfection with GFP DNA as well as WDR1, WDRΔ35 and mutant GFP fusion genes. After 24 hours the culture insert was removed and video microscopy was used to examine the wound healing process. Fluorescent and bright field images of cells were taken every 10 minutes over a period of 24 hours. Fig.3.3 shows bright field and fluorescent merged images of cells that were transfected with pEGFP (3.3A), pEWDR1 DNA (3.3B) and pEWDRΔ35 DNA (3.3C) at time zero and after 24 hours. Moreover, cells were tracked using the image j manual tracking tool and then analyzed using the image j chemotaxis tool to get the average velocity in microns per minute ($\mu\text{M}/\text{min}$) (Fig.3.4). Firstly the mean velocity was found to be $0.465\mu\text{m}/\text{min}$ for the GFP control, $0.343\mu\text{m}/\text{min}$ for cells overexpressing WDR1, and $0.345\mu\text{m}/\text{min}$ for cells overexpressing WDRΔ35 (Fig.3.4). Overall, it was found that WDR1 and WDRΔ35 both caused an extremely significant decrease in cell migration compared to the GFP control ($p<0.0001$). There was no significant difference in velocity between WDR1 and WDRΔ35 ($p>0.05$) (Fig.3.4).

Similarly, HEK293 cells were overexpressed with the various WDR1 and WDRΔ35 mutants to determine whether these mutations may have been introduced in residues that are important in the functional role of WDR1 and or WDRΔ35 in cell

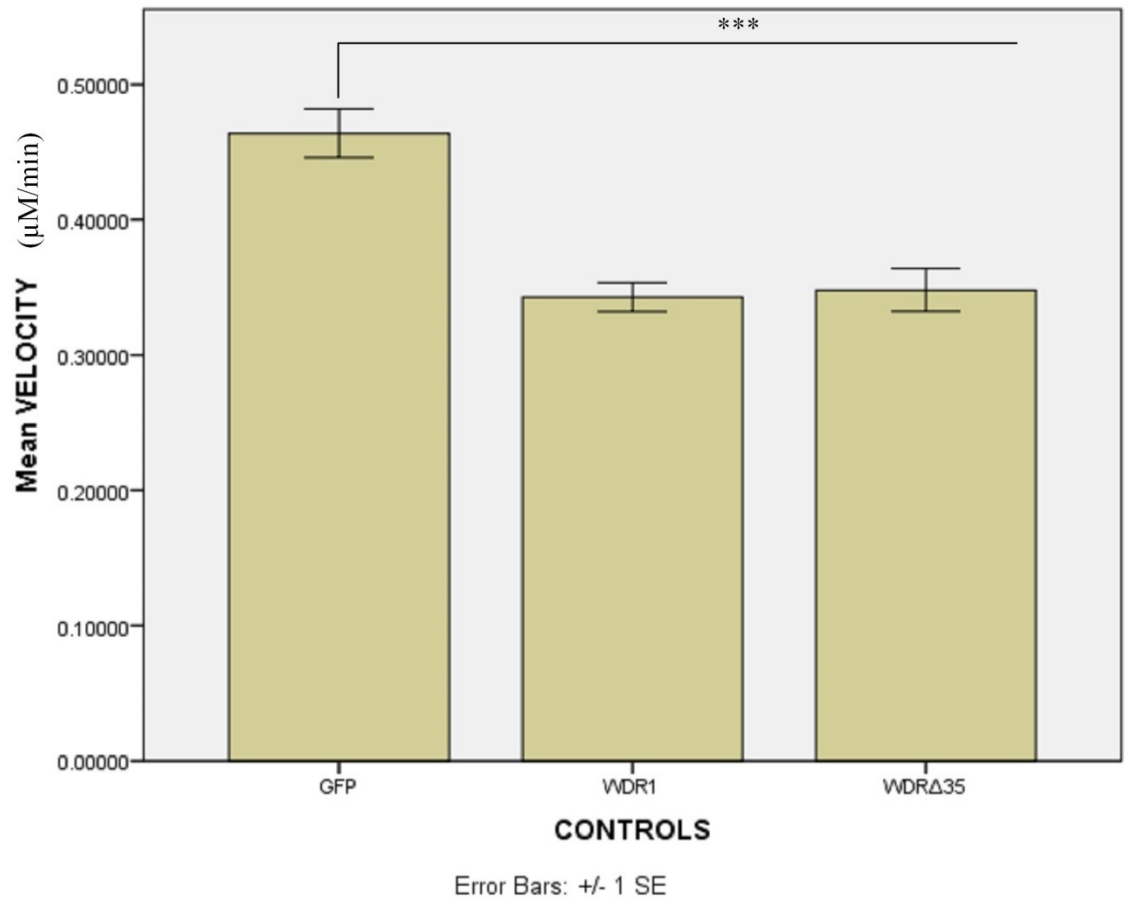


Figure 3.4: WDR1 and WDRΔ35 both caused a significant decrease in cell migration compared to the GFP control. HEK293 cells were plated in ibidi culture inserts 24 hours prior to transfection with GFP DNA as well as WDR1 and WDRΔ35 GFP fusion genes. Next, 24 hours after transfection the culture insert was removed and video microscopy was used to examine the wound healing process. Cells were tracked using the image j manual tracking tool and then analyzed using the image j chemotaxis tool to get the average velocity in microns per hour ($\mu\text{M}/\text{min}$). WDR1 and WDRΔ35 both cause a significant decrease in cell migration compared to the GFP control. There is no significant difference in velocity between WDR1 and WDRΔ35. *** $P < 0.0001$.

migration. Overall, the mean velocity was found to be 0.31 $\mu\text{m}/\text{min}$ for cells overexpressing WDRR17G or WDRG299E, 0.3 $\mu\text{m}/\text{min}$ for cells overexpressing WDRH188Q or WDRW602STOP, and 0.29 $\mu\text{m}/\text{min}$ for cells overexpressing WDRG344E or WDRH531Y (Fig.3.5). WDR1 and all of the WDR1 mutants caused a significant decrease in cell migration compared to the GFP control ($p < 0.0001$). Importantly, WDRH188Q, WDRG344E, WDRH531Y and WDRW602STOP caused a significant decrease in cell migration compared to WDR1 ($p < 0.05$) which suggests that these mutations were introduced in residues that are critical to the function of WDR1 in cell migration. However, there was no significant difference in velocity between WDR1 and WDRR17G or WDRG299E which implies these mutations were made in regions that are not important in the function of WDR1 ($p > 0.05$) (Fig.3.5).

Furthermore, for the WDR Δ 35 mutants the mean velocity was found to be 0.29 $\mu\text{m}/\text{min}$ for cells overexpressing WDR Δ 35H48Q, 0.32 $\mu\text{m}/\text{min}$ for cells overexpressing WDR Δ 35G159E and 0.26 $\mu\text{m}/\text{min}$ for cells overexpressing WDR Δ 35G204E (Fig.3.6). Overall, WDR Δ 35 and all of the WDR Δ 35 mutants caused a significant decrease in cell migration compared to the GFP control ($p < 0.0001$). Of importance, WDR Δ 35H48Q ($p = 0.0214$) and WDR Δ 35G204E ($p = 0.0007$) both caused a significant decrease in cell migration compared to WDR Δ 35 which suggests that H48 and G204 are located in regions that are important to the function of WDR Δ 35. In contrast, there was no significant difference in velocity between WDR Δ 35 and WDR Δ 35G159E ($p > 0.05$) which implies that G159 is not a critical residue for the function of WDR Δ 35 in cell migration

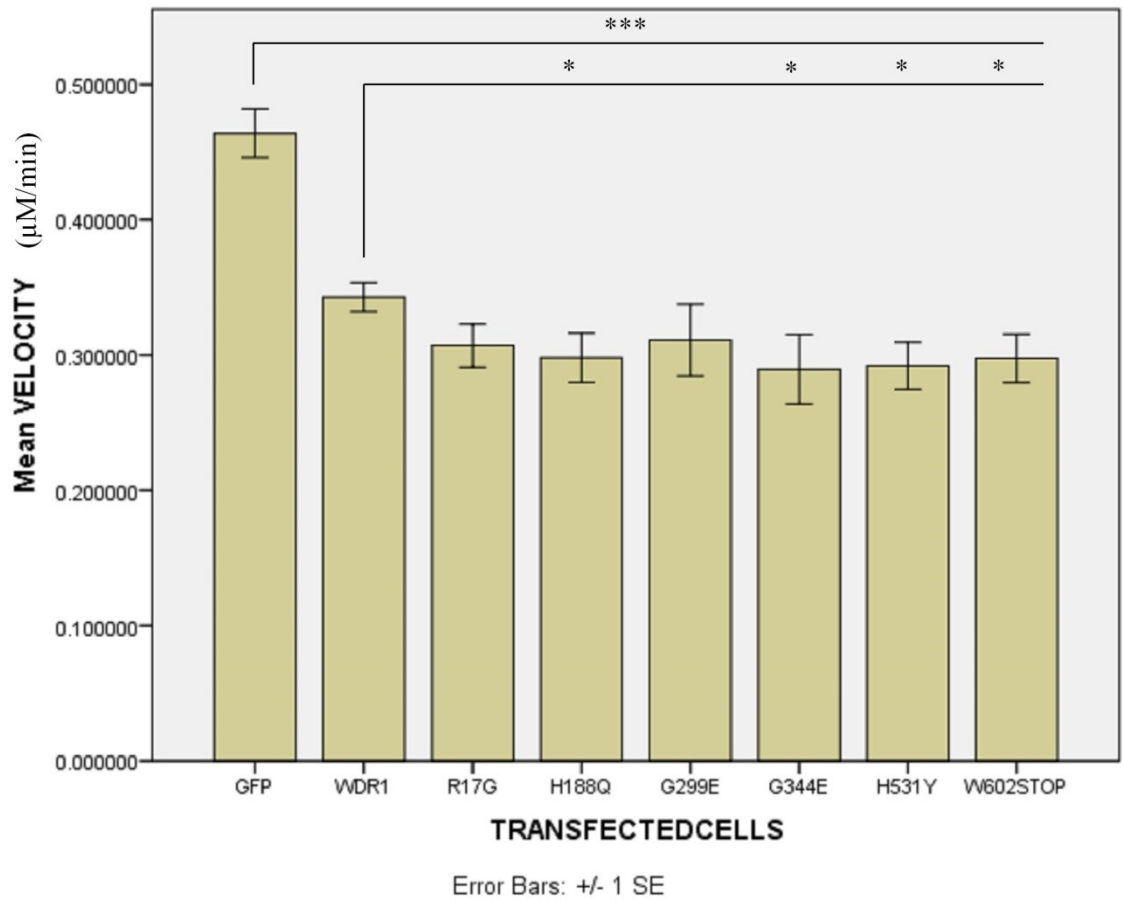


Figure 3.5: WDRH188Q, WDRG344E, WDRH531Y and WDRW602STOP caused a significant decrease in cell migration compared to WDR1 and GFP transfected cells. HEK293 cells were plated in ibidi culture inserts 24 hours prior to transfection with GFP DNA as well as WDR1, WDRR17G, WDRH188Q, WDRG299E, WDRG344E, WDRH531Y and WDRW602STOP GFP fusion genes. Next, 24 hours after transfection the culture insert was removed and video microscopy was used to examine the wound healing process. Cells were tracked using the image j manual tracking tool and then analyzed using the image j chemotaxis tool to get the average velocity in microns per hour ($\mu\text{M}/\text{min}$). WDR1 and all of the WDR1 mutants cause a significant decrease in cell migration compared to the GFP control. WDRH188Q, WDRG344E, WDRH531Y and WDRW602STOP cause a significant decrease in cell migration compared to both WDR1 and GFP transfected cells. There is no significant difference in velocity between the WDR1 mutants or between WDR1 and WDRR17G or WDRG299E. * $P < 0.05$; *** $P < 0.0001$.

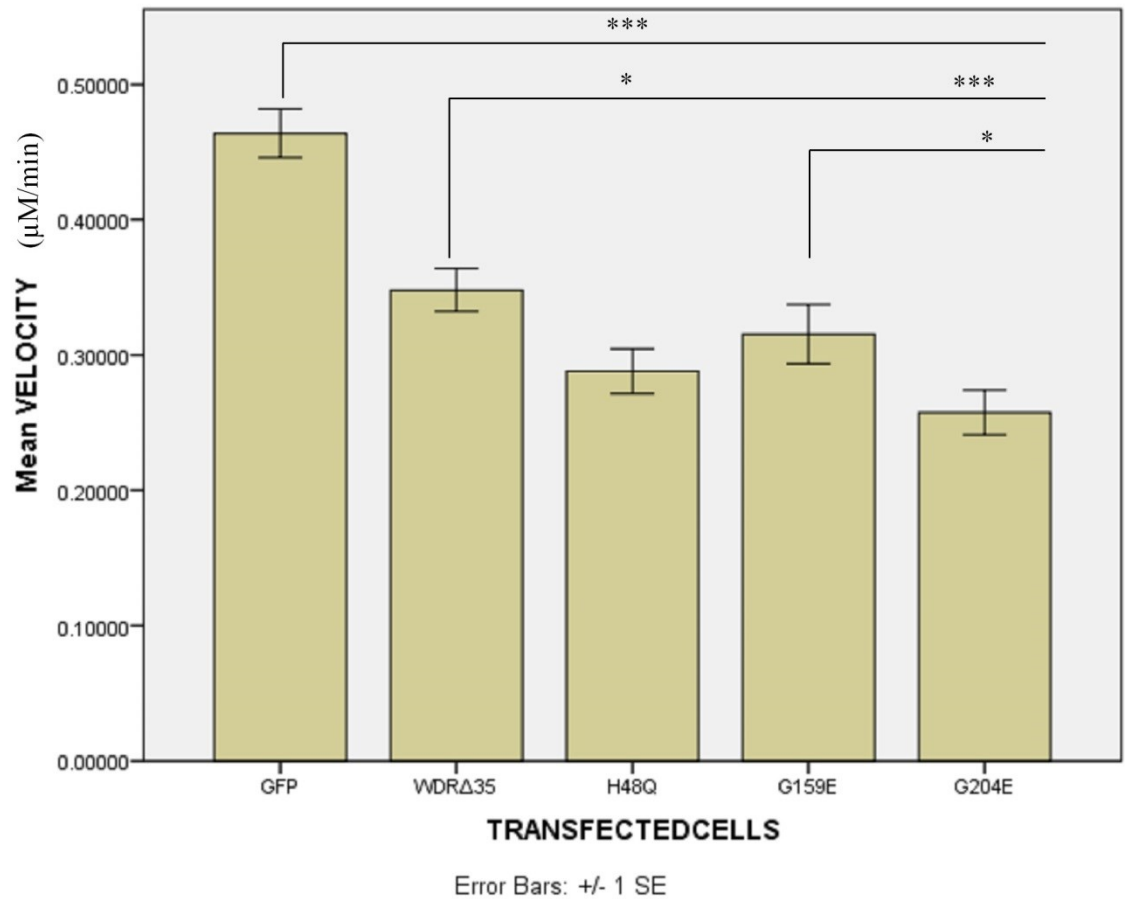


Figure 3.6: WDRΔ35H48Q and WDRΔ35G204E caused a significant decrease in cell migration compared to WDRΔ35 and GFP transfected cells. HEK293 cells were plated in ibidi culture inserts 24 hours prior to transfection with GFP DNA as well as WDRΔ35, WDRΔ35H48Q, WDRΔ35G159E, WDRΔ35G204E GFP fusion genes. Next, 24 hours after transfection the culture insert was removed and video microscopy was used to examine the wound healing process. Cells were tracked using the image j manual tracking tool and then analyzed using the image j chemotaxis tool to get the average velocity in microns per hour (μM/min). WDRΔ35 and all of the WDRΔ35 mutants cause a significant decrease in cell migration compared to the GFP control. WDRΔ35H48Q and WDRΔ35G204E both cause a significant decrease in cell migration compared to WDRΔ35 and GFP transfected cells. WDRΔ35G204E causes a significant decrease in cell migration compared to WDRΔ35G159E. There is no significant difference in velocity between WDRΔ35 and WDRΔ35G159E. There is no significant difference in velocity between WDRΔ35H48Q and WDRΔ35G159E or WDRΔ35G204E. *P<0.05; ***P<0.0001.

Actin turnover rates of WDR1, WDRΔ35 and the described mutants

To determine if actin turnover rates in cells that were overexpressing GFP-tagged WDR1, WDRΔ35 could be affecting cell migration rates, the ratios of F/G actin were quantified using an assay developed by Cytoskeleton Inc. Previous studies have revealed that WDR1 enhances cofilin's activity in severing actin filaments which in turn replenishes G-actin monomers for further rounds of polymerization and when WDR1 is knocked down there is an increase in filamentous actin and an overall decrease in cell migration (Kato, 2008). Thus, in order to determine the ratio of F-actin/G-actin in the cell, HEK293 cells were transfected with GFP, and WDR1 and WDRΔ35 GFP fusion genes. After 24 hours cells were lysed and processed into supernatant (S) (G-actin) and pellet (P) (F-actin) fractions. Samples were run on a western and analyzed using densitometry. Overall, the mean F/G actin ratio was found to be 0.121 for the GFP control, 0.023 for cells overexpressing WDR1, and 0.016 for cells overexpressing WDRΔ35 (Fig.3.7 A, B). Interestingly, both WDR1 ($p<0.05$) and WDRΔ35 ($p<0.01$) had significantly lower F/G ratios compared to the GFP control which implies more G-actin actin monomers being produced and thus higher actin turnover rates in these cells (Fig. 3.7B). There was no significant difference between the F/G ratio of WDR1 and WDRΔ35 ($p>0.05$) which suggests they are having similar effects on actin turnover rates in HEK293 cells (Fig.3.7B). Moreover, as a positive control, HEK293 cells were treated with $0.1\mu\text{M}$ of the actin polymerizing drug jasplakinolide for one hour and the mean F/G actin ratio was found to be 687.3 which was significantly higher than cells overexpressed with WDR1, WDRΔ35 or GFP alone ($p<0.0001$) (Fig.3.7C). Therefore, cells that were treated with jasplakinolide had higher amounts of filamentous actin which showed the assay was

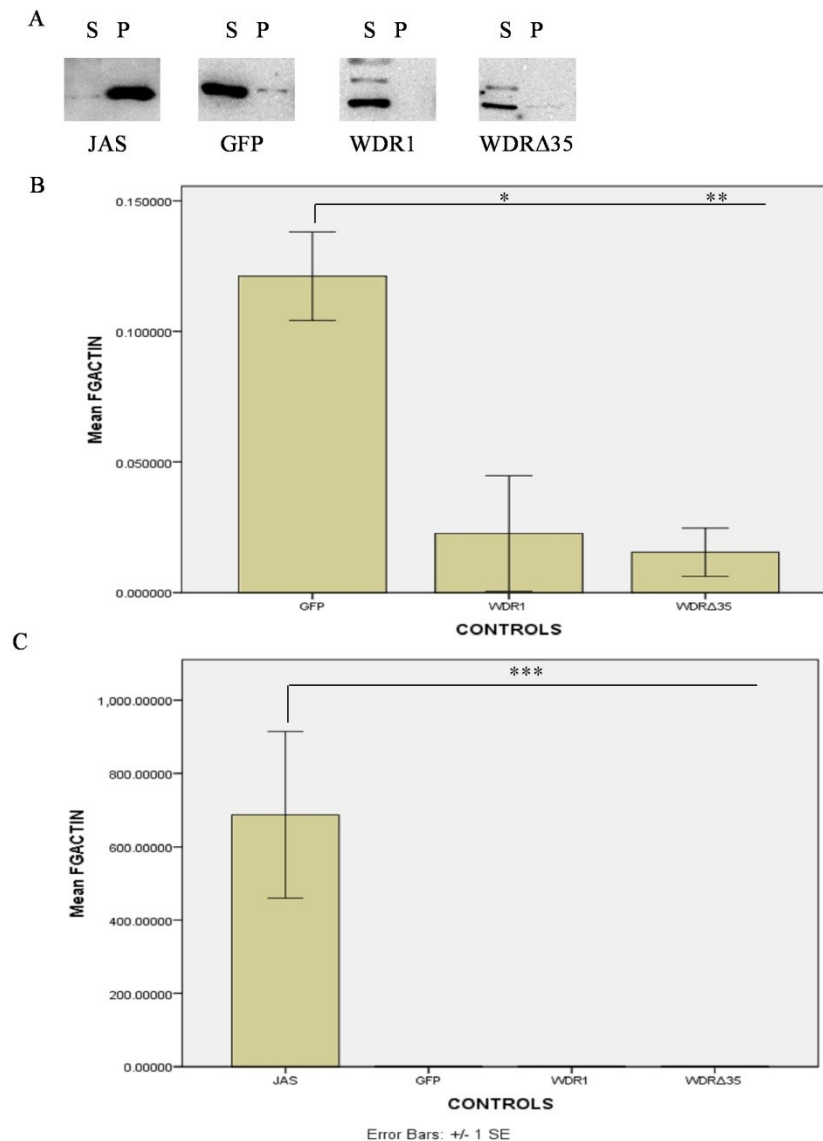


Figure 3.7: WDR1 and WDRΔ35 had a significantly lower ratio of F-actin to G-actin (F/G ratios) compared to the GFP control. HEK293 cells were transfected with GFP, and WDR1 and WDRΔ35 GFP fusion genes. After 24 hours cells were lysed and processed into supernatant (S) and pellet (P) fractions. Samples were run on a western and analyzed using densitometry. A) Immunoblot of actin in cell lysates of cells transfected with GFP, WDR1 and WDRΔ35 GFP fusion genes. Immunoblot of actin in cell lysate of cells treated with 0.1 μ M of the actin polymerizing drug jasplakinolide. B) F/G ratio from densitometry of actin bands. WDR1 and WDRΔ35 have significantly lower F/G ratios compared to the GFP control. There is no significant difference between the F/G ratio of WDR1 and WDRΔ35. C) F/G ratio from densitometry of actin bands. Cells treated with jasplakinolide have significantly higher F/G ratios compared to cells overexpressing GFP, WDR1 and WDRΔ35. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

functioning properly.

Furthermore, the actin turnover rates were also examined in cells overexpressing the WDR1 mutants to determine whether they were introduced in active functional domains of WDR1 (Fig.3.8A, B). The mean F/G actin ratio was found to be 0.396 for cells overexpressing WDRR17G, 0.079 for cells overexpressing WDRH188Q, 0.183 for cells overexpressing WDRG299E, 0.260 for cells overexpressing WDRG344E, 0.260 for cells overexpressing WDRH531Y, and 0.039 for cells overexpressing WDRW602STOP (Fig.3.8B). Of interest, WDRR17G had a significantly higher F/G ratio in comparison to WDR1 ($p < 0.05$) which indicates higher amounts of F-actin being produced in the cell and lower actin turnover rates. Overall, this result suggests that R17G may be an important residue in the function of WDR1 in promoting actin filament turnover in cells. In contrast, no significant difference was found in the F/G actin ratio between WDR1 and WDRH188Q, WDRG299E, WDRG344E, WDRH531Y or WDRW602STOP which suggests that these mutations were introduced into nonfunctional domains of WDR1.

Moreover, when looking at the actin turnover rates in cells overexpressing the GFP-tagged WDR Δ 35 mutants the mean F/G actin ratio was found to be 0.298 for cells overexpressing WDR Δ 35H48Q, 0.007 for cells overexpressing WDR Δ 35G159E, and 0.437 for cells overexpressing WDR Δ 35G204E (Fig.3.9A,B). Overall, both WDR Δ 35H48Q ($p < 0.05$) and WDR Δ 35G204E ($p < 0.01$) had significantly higher F/G actin ratios in comparison to WDR Δ 35 which shows there are higher amounts of F-actin being produced in these cells and lower actin turnover rates (Fig.3.9B). Thus, this result

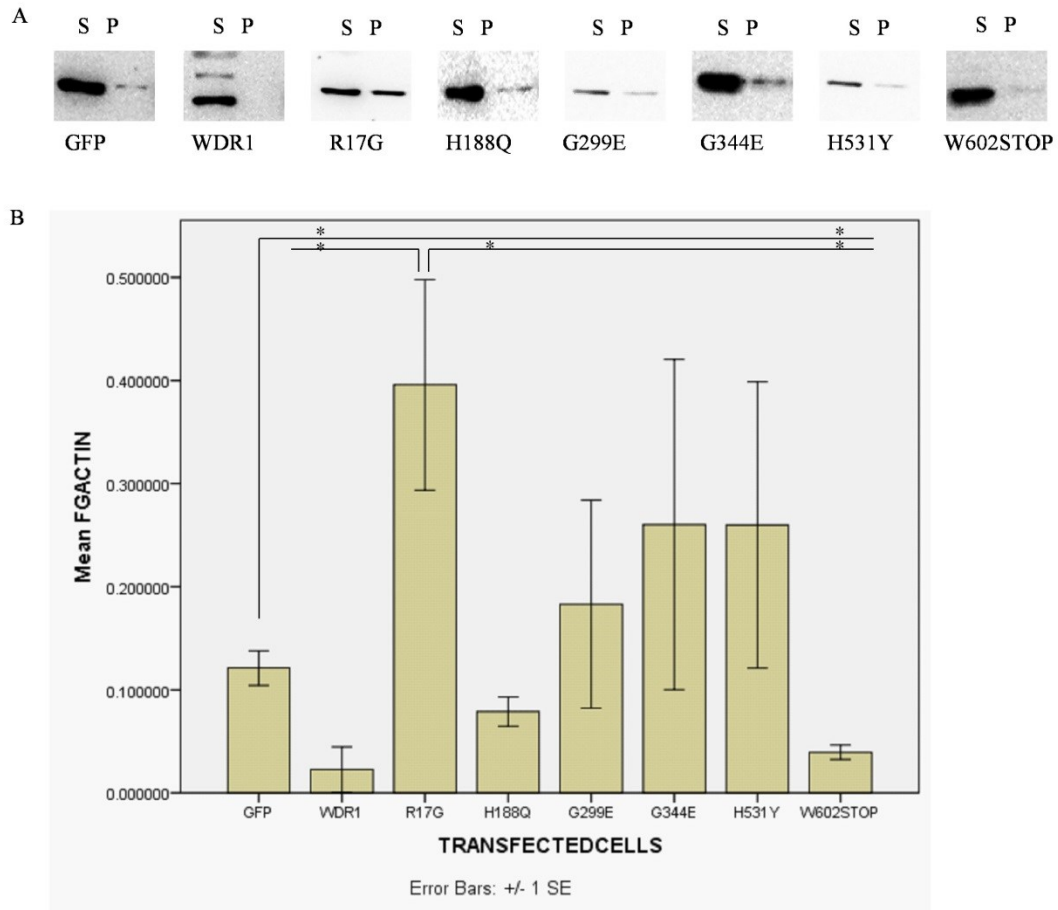


Figure 3.8: WDRR17G had a significantly higher ratio of F-actin to G-actin (F/G ratios) compared to WDR1. HEK293 cells were transfected with GFP, and WDR1, WDRR17G, WDRH188Q, WDRG299E, WDRG344E, WDRH531Y, and WDRW602STOP GFP fusion genes. After 24 hours cells were lysed and processed into supernatant (S) and pellet (P) fractions. Samples were run on a western and analyzed using densitometry. A) Immunoblot of actin in cell lysates of cells transfected with GFP, and WDR1, WDRR17G, WDRH188Q, WDRG299E, WDRG344E, WDRH531Y, and WDRW602STOP GFP fusion genes. B) F/G ratio from densitometry of actin bands. WDRR17G has a significantly higher F/G actin ratio compared to WDR1, WDRH188Q, and WDRW602STOP. Both WDR1 and WDRW602STOP have a significantly lower F/G actin ratio compared to the GFP control. There is no significant difference in the F/G actin ratios between the GFP control and WDRR17G, WDRH188Q, WDRG299E, WDRG344E and WDRH531Y. There is no significant difference in the F/G actin ratio between WDR1 and WDRH188Q, WDRG299E, WDRG344E, WDRH531Y and WDRW602STOP. * $P < 0.05$.

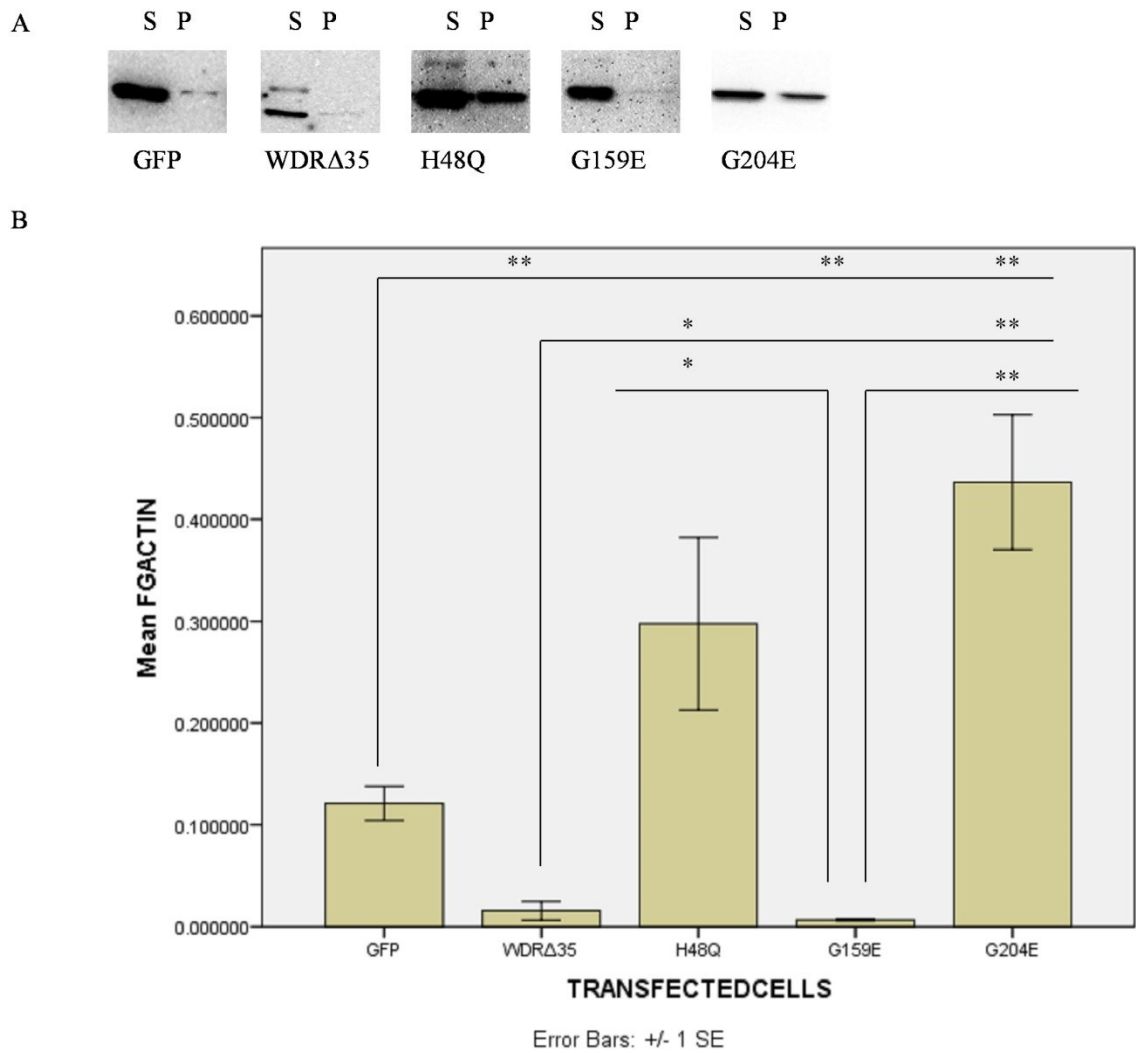


Figure 3.9: WDRΔ35H48Q and WDRΔ35G204E had significantly higher ratios of F-actin to G-actin (F/G ratios) compared to WDRΔ35. HEK293 cells were transfected with GFP, and WDRΔ35, WDRΔ35H48Q, WDRΔ35G159E and WDRΔ35G204E GFP fusion genes. After 24 hours cells were lysed and processed into supernatant (S) and pellet (P) fractions. Samples were run on a western and analyzed using densitometry. A) Immunoblot of actin in cell lysates of cells transfected with GFP, and WDRΔ35, WDRΔ35H48Q, WDRΔ35G159E and WDRΔ35G204E GFP fusion genes. B) F/G ratio from densitometry of actin bands. WDRΔ35 and WDRΔ35G159E both have significantly lower F/G actin ratios compared to the GFP control. WDRΔ35G204E has a significantly higher ratio of F-actin to G-actin compared to WDRΔ35, WDRΔ35G159E and the GFP control. WDRΔ35H48Q has a significantly higher F/G actin ratio compared to WDRΔ35 and WDRΔ35G159E. There is no significant difference in the F/G ratios between WDRΔ35 and WDRΔ35G159E. * $P < 0.05$; ** $P < 0.01$.

shows that the amino acid residues H48 and G204 may be critical to the function of WDR Δ 35 in regulating actin cytoskeletal rearrangements. Moreover, there was no significant difference in the ratio of F/G actin in cells that were overexpressed with WDR Δ 35G159E ($p>0.05$) compared to WDR Δ 35 which suggests that this residue is not found in a functional region of WDR Δ 35 that is important in enhancing the turnover of the actin cytoskeleton (Fig.3.9B).

Investigate potential interacting partners of WDR1 and WDR Δ 35

Previous studies in yeast have shown that Aip1p can interact with actin (Rodal, 1999) but it is not known if AIP1/WDR1 interacts with actin in mammals. Thus, in order to see if WDR1 or WDR Δ 35 are interacting with actin or other actin binding proteins (e.g. cofilin and CAP1), HEK293 cells were overexpressed with WDR1 and WDR Δ 35 GFP fusion genes and a GFP control and then lysed and collected after 24 hours. Co-immunoprecipitation was performed with cell lysates and GFP trap beads. Supernatant (S) and pellet (P) samples were then run on a western. The pellet samples contained immunocomplexes made up of a GFP fusion protein and any interacting factors whereas the supernatants contained proteins that are not interacting with the GFP fusion protein. Samples were then run on a western and probed using anti-GFP and anti-actin antibody to see if WDR1 and or WDR Δ 35 are interacting with actin. Overall, actin was found in the supernatants of both WDR1 and WDR Δ 35 and therefore WDR1 and WDR Δ 35 do not interact with actin under the conditions applied (Fig.3.10). The experiment was repeated and samples were then run on a western and probed using anti-GFP, anti-actin, anti-CAP1, and anti-cofilin antibody to see if WDR1 and or WDR Δ 35 are interacting with

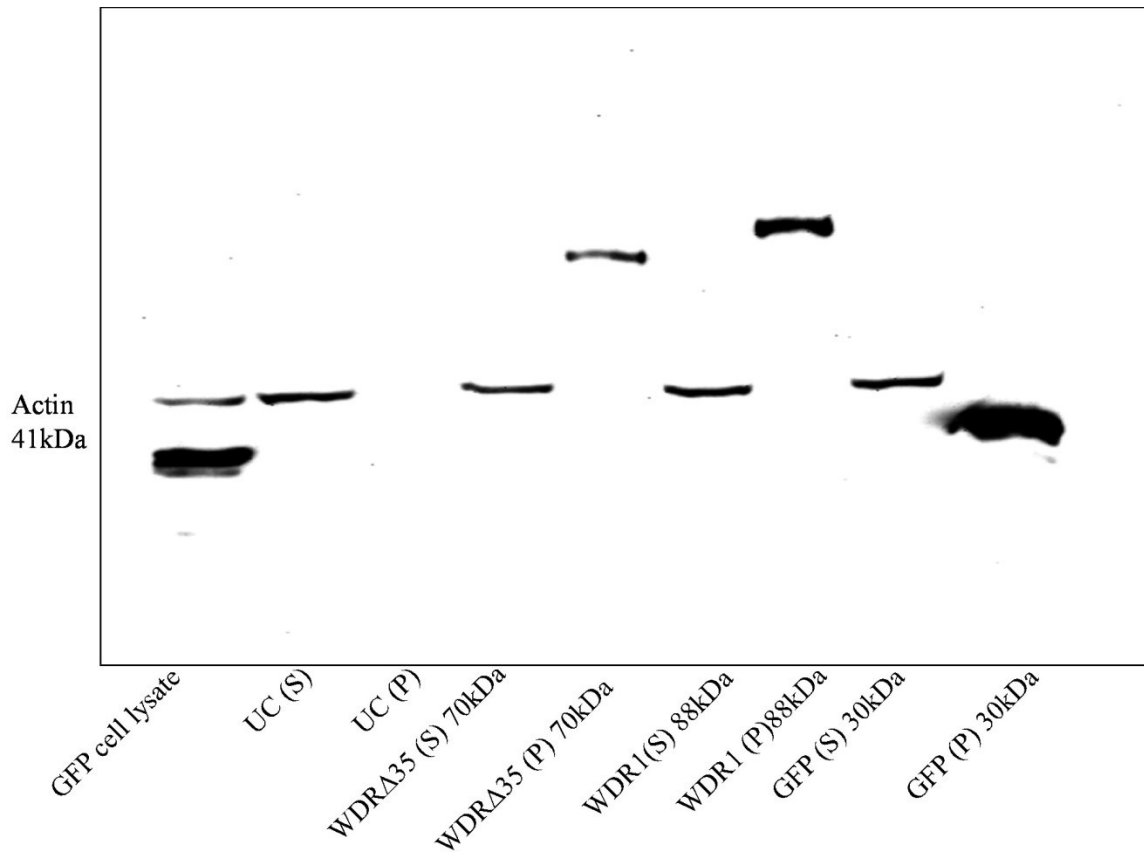


Figure 3.10: WDR1 and WDRΔ35 did not interact with actin under the conditions applied. HEK293 cells were overexpressed with WDR1 and WDRΔ35 GFP fusion genes and a GFP control and then lysed and collected after 24 hours. Co-immunoprecipitation was performed with cell lysates and GFP trap beads. Supernatant (S) and pellet (P) samples were then run on a western. The pellet samples contain immunocomplexes made up of a GFP fusion protein and any interacting factors whereas the supernatants contain proteins that are not interacting with the GFP fusion protein. UC represents the untransfected negative control. The GFP cell lysate is a positive control for actin. Samples were then run on a western and probed using anti-GFP and anti-actin antibody to see if WDR1 and or WDRΔ35 are interacting with actin. Actin 42kDa is present in both the supernatants of WDR1 and WDRΔ35 and so they do not interact with actin under the conditions applied.

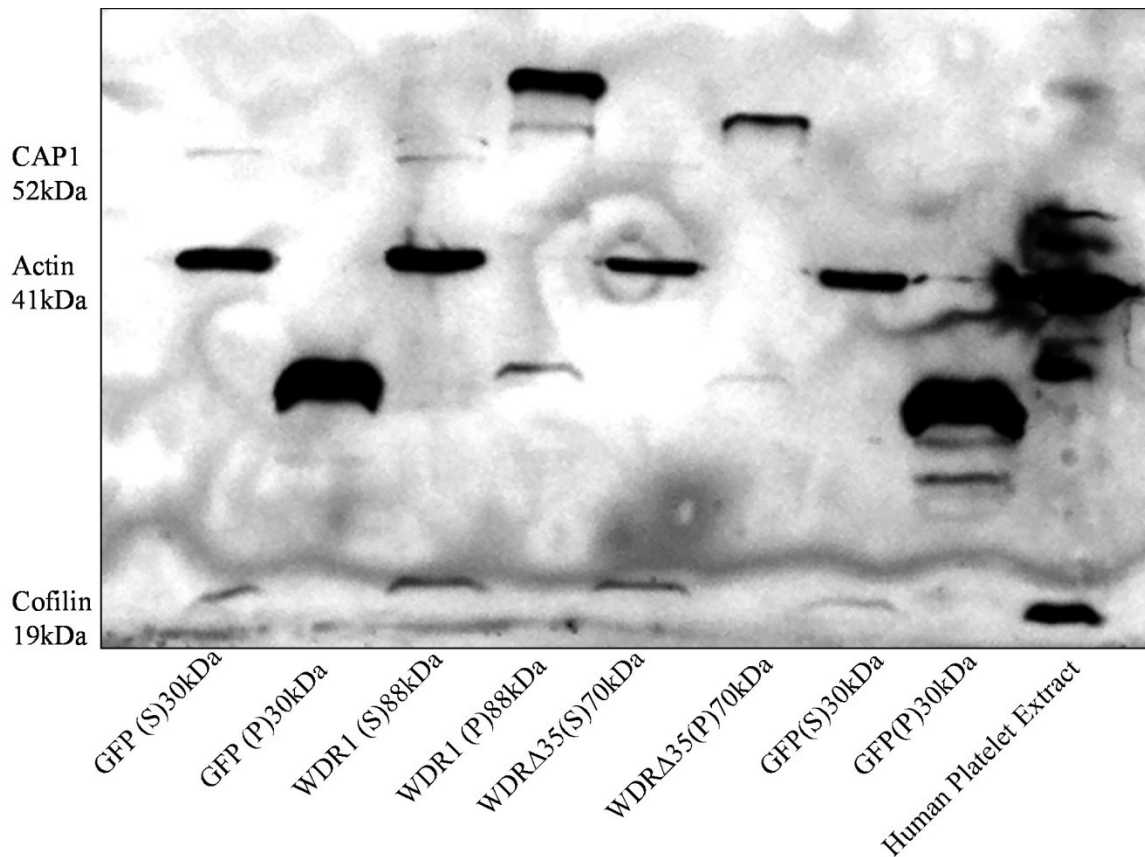


Figure 3.11: WDR1 and WDRΔ35 did not interact with actin, CAP1 or cofilin under the conditions applied. HEK293 cells were overexpressed with WDR1 and WDRΔ35 GFP fusion genes and a GFP control and then lysed and collected after 24 hours. Co-immunoprecipitation was performed with cell lysates and GFP trap beads. Supernatant (S) and pellet (P) samples were then run on a western. The pellet samples contain immunocomplexes made up of a GFP fusion protein and any interacting factors whereas the supernatants contain proteins that are not interacting with the GFP fusion protein. The human platelet extract is a positive control for cofilin. The Samples were then run on a western and probed using anti-GFP, anti-actin, anti-CAP1, and anti-cofilin antibody to see if WDR1 and or WDRΔ35 are interacting with actin, CAP1 or cofilin. Actin 42kDa, CAP1 52kDa and cofilin 19kDa are present in both the supernatants of WDR1 and WDRΔ35 and so they do not interact with them under the conditions applied.

actin, CAP1 or cofilin (Fig.3.11), proteins that have been implied or shown in some organisms to interact with WDR1 (Moriyama, 2002; Balcer, 2003). Overall, it was found that actin, CAP1 and cofilin were present in both the supernatants of WDR1 and WDR Δ 35 but failed to immunoprecipitate with GFP-WDR1 and WDR Δ 35 under the conditions applied (Fig.3.11).

Summary of the velocity and F/G actin results for WDR1 and WDRΔ35 mutants

WDR1 MUTANTS	VELOCITY		F/G ACTIN	
	GFP	WDR1	GFP	WDR1
WDRR17G	↓↓↓			↑
WDRH188Q	↓↓↓	↓		
WDRG299E	↓↓↓			
WDRG344E	↓↓↓	↓		
WDRH531Y	↓↓↓	↓		
WDRW602STOP	↓↓↓	↓	↓	
WDRΔ35 MUTANTS	GFP	WDRΔ35	GFP	WDRΔ35
WDRΔ35H48Q	↓↓↓	↓		↑
WDRΔ35G159E	↓↓↓		↓↓	
WDRΔ35G204E	↓↓↓	↓↓↓	↑↑	↑↑

Fig. 3.12 Summary of the velocity and F/G actin results for the WDR1 and WDRΔ35 mutants.

CHAPTER 4

DISCUSSION

Actin is a highly conserved eukaryotic protein that is the major constituent of the actin cytoskeleton which plays an important role in a wide range of biological processes such as cell migration, endocytosis and cytokinesis (Kato, 2008). Moreover, there are numerous actin binding proteins that exist in the cell and serve to regulate the dynamics and reorganization of the actin cytoskeleton through processes such as nucleation, elongation, severing, crosslinking and stabilizing F-actin in a cell (Thomas, 2012; Pollard, 2000; Winder, 2005). Misregulation of actin binding proteins can give rise to cancer where often times they are overexpressed which gives malignant cancer cells their ability to invade healthy tissues in the body (Yamaguchi, 2007). Therefore, it is beneficial to study the functional role of actin binding proteins in regulating the actin cytoskeleton because it can give valuable insight into ways to prevent malignant cancer cells from migrating and invading nearby tissues. The actin binding protein that was studied in this thesis was WDR1 which is also known as Actin Interacting Protein 1 (AIP1) in lower eukaryotes. Very little is known about the function of WDR1/AIP1 except that it has been shown to enhance cofilin's ability to sever and depolymerize actin filaments (Kato, 2008; Ono, 2003; Ono, 2007; Izawa, 2013). Moreover, our lab discovered a splice variant of WDR1 termed WDR Δ 35 which is lacking exons 3-5 as well as 3 of the 9 WD repeats found in WDR1 (Fig.3.1, A, B) which could have functional consequences since WD repeats are known to play an important role in mediating protein-protein interactions (Tousignant, R. et al., manuscript in preparation; Luo, 2013; Smith, 1999). Studies have shown that WDR1 is upregulated in breast cancer and ovarian cancer tissues where it may contribute to the invasive properties observed in these malignant cells through its ability to increase actin turnover rates with cofilin leading to an

increase in directed cell migration and invasion (Haslene-Hox, 2013; Kang, 2010). In contrast, nothing is known about the functional role of WDR Δ 35 in regulating the actin cytoskeleton or the effects that it may have on cell migration. The main purpose of this study was to study the effect of the overexpression of WDR1 and WDR Δ 35 on cell migration in mammalian cells. Furthermore, to study potential functional domains within the two proteins, a series of site directed mutations were expressed and monitored for cell migration. In addition, the ratio of G-actin/F-actin was measured upon WDR1/WDR Δ 35 perturbation in an attempt to quantify the effects on cell migration with the ability to control actin turnover in cells.

WDR1 and WDR Δ 35 caused a significant decrease in cell migration and had significantly lower ratios of F-actin to G-actin compared to the GFP control.

It was found that WDR1 and WDR Δ 35 both caused a significant decrease in cell migration compared to the GFP control which suggests they may play an important role in cell migration (Fig.3.4). This result is contradictory to other studies that have shown that the upregulation of WDR1 in breast and ovarian cancer tissues may contribute to the higher actin turnover rates in these cells through enhancing cofilin's actin filament depolymerizing activity and causing an overall increase in cell migration and invasion rates (Haslene-Hox, 2013; Kang, 2010). Whereas, when WDR1 is knocked down in Jurkat cells, this caused an increase in F-actin filaments leading to the formation of multiple cellular protrusions and a decrease in directed cell migration (Kato. 2008). Overall, both scenarios demonstrated the role of WDR1 in promoting cofilin's ability to sever and depolymerize actin filaments where its upregulation caused an increase in turnover of actin filaments and cell migration and it's down regulation caused a decrease in turnover of F-actin and cell migration. Thus, to examine actin turnover rates in cells that were transfected with GFP tagged WDR1 and WDR Δ 35, an F-actin to G-actin (F/G

ratio) kit was used to separate G-actin in the supernatant from F-actin in the pellet. Examining the levels of filamentous actin to monomeric actin in the cell made it possible to determine if the overexpression of WDR1 and or WDR Δ 35 are causing a decrease in cell migration via changing actin filament turnover rates in cells. Overall, it was found that both WDR1 and WDR Δ 35 had a significantly lower ratio of F-actin to G-actin compared to the GFP control (Fig.3.7). This result demonstrates that both WDR1 and WDR Δ 35 were generating higher amounts of G-actin monomers in cells which is agreeable with previous studies that show WDR1's role in enhancing cofilin's activity in severing and depolymerizing actin filaments and thus generating free actin monomers for further rounds of polymerization (Kato, 2008; Ono, 2003; Ono, 2007; Izawa, 2013). Interestingly, WDR1 and WDR Δ 35 had similar effects on increasing G-actin monomers in HEK293 cells and decreasing cell migration rates (Fig.3.4, Fig.3.7). One of the main objectives of this study was to study any differences in functional roles between WDR1 and WDR Δ 35 in mammalian cells. From an evolutionary perspective, there should be a functional reason for the presence of a differentially spliced WDR Δ 35 isoform. Previous studies in our lab have shown that WDR1 and WDR Δ 35 were expressed at different levels in different cells and tissues and WDR Δ 35 was expressed at higher levels in the liver, brain, testis and the breast (Tousignant, R. et al., manuscript in preparation). Interestingly, this result agrees with other studies that have revealed that alternative splicing occurs most frequently in complex tissues such as the brain and testis that contain a variety of cell types (Roy, 2013; Blencowe, 2006). Moreover, when cofilin was overexpressed in HEK293 cells there was a significant upregulation in WDR Δ 35 levels and a down regulation in WDR1 levels which suggests a potential linkage between

WDR Δ 35 and cofilin and signalling pathways (Correa, R., et al., manuscript in preparation). It is known that alternative splicing that leads to the formation of a truncated isoform can result in the splice variant acting as a dominant negative of the wild-type protein where it can interact and inhibit its interactions with protein substrates (Staiger, 2013; Seo, 2011). One completely theoretical explanation is that WDR Δ 35 may be acting as a dominant negative with WDR1 in more functionally complex tissues such as the brain where it may interact with WDR1 and alter its conformation so that it can no longer interact with cofilin and enhance its activity. Alternatively it may be binding different regulatory proteins (due to its missing WD domains) and sequester them away from WDR1. This could serve as a regulatory mechanism when cofilin levels are too high in the cell. For example, it was found that when HEK293 cells were overexpressed with WDR1 or WDR Δ 35 there was an increase in G-actin monomers (Fig.3.7B) which is consistent with the concept that WDR1 enhances cofilin's actin filament severing activity (Kato, 2008; Ono, 2003; Ono, 2007; Izawa, 2013) as well as a decrease in cell migration (Fig.34) which contrasts other studies that show the upregulation of WDR1 is linked to an increase in cell migration (Haslene-Hox, 2013; Kang, 2010). However, in order for cell migration to take place cells must undergo coordinated cycles of assembly and disassembly where actin monomers polymerize at the cell's leading edge forming membrane protrusions that drive the cell in a specific direction as well as depolymerize so that G-actin monomers are made available for another round of polymerization (Bailly, 2007). Moreover, although cofilin is known to increase actin turnover rates by severing actin filaments and making free actin monomers available for polymerization, it can also have inhibitory effects through its ability to bind G-actin and prevent the nucleotide exchange of ADP for ATP which is required for polymerization (Moriyama, 2002;

Nishida, 1985; Blanchoin, 1998). Also, phosphorylation of cofilin by protein kinases such as LIM kinases releases ADP-actin monomers so they can be converted to ATP-actin which is competent for polymerization (Moriyama, 2002). In addition, studies have shown that the C-terminal domain of CAP1 interacts with cofilin and stimulates the nucleotide exchange on G-actin (Moriyama, 2002). Thus, this may explain why the overexpression of WDR1 and WDR Δ 35 caused a decrease in cell migration (Fig.3.4). For instance, WDR1 and WDR Δ 35 may be enhancing cofilin's activity in severing actin filaments to produce G-actin monomers (Fig.3.7) which causes cofilin to become over-activated where it is not inhibited by protein kinases and or there is not enough CAP1 to reduce cofilin's inhibitory effects on nucleotide exchange on G-actin monomers. Overall, this would result in a decrease in cell migration (Fig.3.4) because there is a pool of ADP-actin monomers (Fig.3.7) in the cell which are not competent for polymerization.

Mutational analysis defined potential active functional domains in both WDR1 and WDR Δ 35

Mutational analysis was used to define potential active functional domains in both WDR1 and WDR Δ 35. In 2003 the crystal structure of AIP1 was determined in yeast which revealed some key residues that are conserved and could be important to the function of AIP1 (Voegtli, 2003; Ono, 2001). From this information site-directed mutagenesis was performed to introduce point mutations in both WDR1 (WDRR17G, WDRH188Q, WDRG299E, WDRG344E, WDRH531Y, and WDRW602STOP) and WDR Δ 35 (WDR Δ 35H48Q, WDR Δ 35G159E, WDR Δ 35G204E) in some conserved residues (Fig.3.1, C-D) which may be located in potential active functional domains of WDR1 and or WDR Δ 35. Next WDR1, WDR Δ 35 as well as the different mutants were overexpressed

as GFP fusion proteins in HEK293 cells (Fig.3.2, A-E) and proteins from these cells were collected and run on a western blot to check that they were stable and being expressed. Overall, the presence of bands at 88kDa for WDR1 and the WDR1 mutants fused to GFP as well as at 70kDa for WDR Δ 35 and the WDR Δ 35 mutants confirmed they were stable and not being degraded in the cell (Fig.3.2A-E). Also cells were examined using fluorescent microscopy to confirm the cells were expressing the mutant GFP-tagged protein (Fig.3.3) Next, the effects of the various mutants on cell migration were monitored using standard assays (Fig.3.5,3.6).

Effects of WDR1 mutants on cell migration

Firstly, it was found that like WDR1, all of the WDR1 mutants which include WDRR17G, WDRH188Q, WDRG299E, WDRG344E, WDRH531Y, and WDRW602STOP caused a significant decrease in cell migration compared to the GFP control (Fig.3.5). Moreover, WDRH188Q, WDRG344E, WDRH531Y and WDRW602STOP caused a significant decrease in cell migration compared to both WDR1 and GFP transfected cells which suggests that these residues are located in regions of WDR1 that are important to its function (Fig.3.5). Interestingly, all four of these mutations were made in regions that contain WD repeats such as WDRH188Q located in WD4, WDRG344E located in WD6, WDRH531Y located in WD8 and WDRW602STOP located in WD9 (Fig.1A,C). Moreover, a study in *C.elegans* that made point mutations in some of these conserved residues (G346E, H535Y and W607STOP) showed that this caused motility defects due to a disorganization of the actin cytoskeleton in the body wall muscle (Ono, 2001). Thus, WDRH188Q, WDRG344E, WDRH531Y and WDRW602STOP may be decreasing cell migration rates via changing actin turnover

rates in mammalian cells. A possible explanation for the outcome in this study is that the mutants could be having various effects on the interactions between WDR1 and its protein substrates which in turn could alter actin turnover rates in cells. For instance, if a point mutation introduced in WDR1 enhanced the affinity between WDR1 and cofilin, this could result in a higher pool of ADP-actin that is not competent for polymerization and a further decrease in cell migration. In contrast, if they lower or restrict the binding affinity of cofilin to WDR1 then this could cause a build-up of F-actin which could also decrease cell migration rates. Therefore, the next step was to examine the actin turnover rates in these cells to uncover if these mutations are altering the effect of WDR1 on cell migration rates by affecting actin turnover rates in cells (Fig.3.8A, B). Firstly, there was no significant difference in the F/G actin ratios of WDR1 and WDH188Q, WDRG344E, WDRH531Y or WDRW602STOP (Fig.3.8B) although these mutants all caused a significant decrease in cell migration in comparison to WDR1 (Fig.3.5) which suggests that they may be altering cell migration rates by a mechanism other than through increasing or decreasing actin filament turnover rates in cells. Previous studies have revealed protein interactions between cofilin, actin, CAP, AIP1 and profilin in yeast (Drees, 2001). Profilin promotes actin filament polymerization by forming a complex with actin and binding to the barbed end of a filament where it deposits actin (Ding, 2012; Nyman, 2002). Thus, if any of the mutations made in H188, G344, H531 or W602 of WDR1 were to induce a change in the shape of WDR1 so that when it interacts with actin it prevents it from interacting with profilin, this would slow or inhibit actin filament polymerization even though there are G-actin monomers available (Fig.3.8B). Moreover, it was found that WDRG299E had no significant difference in the ratio of F/G actin (Fig.3.8) or in cell migration (Fig.3.5) in comparison to WDR1 implying that this

mutation is probably not causing a marked difference in activity which agrees with the fact that this mutation was not introduced in a WD repeat region (Fig. 3.1C). Moreover, there was no difference in migration rates between WDRR17G and WDR1 (Fig.3.5) but WDRR17G had a significantly higher F/G ratio than WDR1 (Fig.3.8B). WDRR17G may have been introduced in a region of WDR1 that altered its conformation and binding affinity for its protein substrates. One possible explanation for this result could be that WDRR17G may have reduced or inhibited the binding affinity of cofilin to WDR1 which would lower cofilin's actin filament severing activity causing a build-up of unsevered filamentous actin and ultimately an overall decrease in cell migration in comparison to the GFP control (Fig.3.5).

WDRΔ35H48Q and WDRΔ35G204E are found in active functional domains of WDRΔ35

It was found that WDRΔ35 and all of the WDRΔ35 mutants caused a significant decrease in cell migration in comparison to the GFP control (Fig.3.6). Moreover, WDRH48Q and WDRG204E caused a significant decrease in cell migration compared to WDRΔ35 which suggests that they may have been introduced in key residues that are important for the function of WDRΔ35 on cell migration rates (Fig.3.6). To investigate whether these WDRΔ35 mutants were causing changes in velocity by increasing or decreasing actin turnover rates in the cell, the F/G actin ratios were examined. Interestingly, both WDRH48Q and WDRG204E had significantly higher ratios of F/G actin in comparison to WDRΔ35 which suggests that they caused a build-up of F-actin in the cell which decreased cell motility rates (Fig.3.9B). Both of these mutations were made in regions that contain WD repeats where WDRH48Q is located in WD1 and WDRG204E is located

in WD3 of WDR Δ 35 and so they may be altering the interactions of WDR Δ 35 with other interacting partners (Fig.3.1D). For instance, if the mutations H48Q or G204E lowered the binding affinity of WDR Δ 35 for cofilin, then this would reduce cofilin's actin filament severing activity and less G-actin monomers would be available for further rounds of polymerization. Overall, there would be an increase in F-actin (Fig.3.9B) and a decrease in cell migration as the final outcome (Fig.3.6). Moreover, there was no significant difference in the ratio of F/G actin or cell migration between WDR Δ 35 and WDR Δ G159E (Fig.3.9B, 3.6) which suggests that WDR Δ 35G159E is not located in an active functional domain of WDR Δ 35. This may not be surprising since WDR Δ 35G159E was not introduced in a WD repeat region of WDR Δ 35 (Fig.3.1D).

WDR1 and WDR Δ 35 did not interact with actin, CAP1 or cofilin under the lysis conditions applied

Aip1p was originally discovered in yeast where it was found to interact with actin and was later shown to interact with cofilin (Amberg, 1995; Rodal, 1999). Shortly after studies revealed that both CAP1 and Aip1p interact with the cofilin-actin complex (Moriyama, 2002; Balcer, 2003). In addition, a yeast two hybrid experiment revealed protein interactions between cofilin, actin, CAP, AIP1 and profilin (Drees, 2001). Thus, actin was believed to be a good candidate protein to begin with in this study to see if it interacts with WDR1 and or WDR Δ 35. A co-immunoprecipitation experiment was performed to determine whether a GFP tagged version of WDR1 and or WDR Δ 35 could be pulled down with actin by using an anti-GFP antibody. Overall, actin was found to be in the supernatants of both WDR1 and WDR Δ 35 and not in the pellet samples that

contain the immunocompexes made up of WDR1 or WDR Δ 35 and any binding partners (Fig.3.10). Thus, it can be concluded that WDR1 and WDR Δ 35 did not interact with actin under the lysis conditions applied (Fig.3.10). Next, this experiment was repeated to investigate whether WDR1/ WDR Δ 35 interacted with other potential protein substrates such as CAP1 and cofilin. Once again, CAP1 and cofilin were found in the supernatant samples of WDR1 and WDR Δ 35 along with actin and did not show up in the immunocomplexes with GFP-WDR1 or GFP-WDRD35. (Fig.3.11). However, these experiments should be repeated as Co-IP experiments are often difficult to perform effectively since they involve a variety factors that need to be optimized such as the lysis buffer that is being used and type of lysis employed.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

In conclusion, the overexpression of GFP-tagged WDR1 and WDR Δ 35 in HEK293 cells caused a significant decrease in the ratio of F-actin/G-actin as well as a decrease in cell migration in comparison to the GFP control. Therefore, WDR1 and WDR Δ 35 appear to have similar effects on increasing the amount of G-actin monomers in the cell for further rounds of polymerization. However, their overexpression caused a decrease in cell migration rates and further studies should be performed to determine whether these free actin monomers are competent for polymerization. Overall, WDR1 and WDR Δ 35 may play an important role in cell migration by generating higher actin turnover rates in mammalian cells. Site-directed mutagenesis was performed to introduce point mutations in conserved residues that may be important in the interactions of WDR1/WDR Δ 35 with their protein substrates. Overall, it was found that WDRH188Q, WDRG344E, WDRH531Y and WDRW602STOP caused an enhanced decrease in cell migration compared to WDR1 alone. Also, cells expressing WDRR17G had a significantly higher F/G actin ratio in comparison to WDR1. Therefore, this result suggests that these mutations may have been made in functional domains of WDR1 that are important in its control over actin turnover rates or cell migration rates. Also, overexpression of WDRG299E in mammalian cells did not cause a difference in cell migration or actin turnover rates in comparison to WDR1 which suggests that this amino acid region may not have any functional significance for WDR1 function. Furthermore, cells overexpressing WDR Δ 35H48Q and WDR Δ 35G204E had significantly higher F/G

actin ratios as well as an enhanced decrease in cell migration compared to WDR Δ 35 alone. Thus, these mutations may have been introduced in an active domain of WDR Δ 35. Lastly, there was no significant difference observed in cell velocity or F/G actin ratios in cells that were overexpressed with WDR Δ 35G159E compared to WDR Δ 35 which implies this residue is not located in an active functional domain of WDR Δ 35. Overall, the results of this study give valuable insight into residues that may be important in the interaction of WDR1 and or WDR Δ 35 with other proteins and by altering their affinity for them can have various effects on actin turnover rates and cell motility rates in cells.

Future Directions

WDR1 and WDR Δ 35 were shown in this study to have similar effects on decreasing cell migration rates and increasing actin turnover rates in cells. Therefore, WDR1 and WDR Δ 35 may have similar roles in enhancing cofilin's actin filament severing activity to break down F-actin and produce G-actin monomers. However, if other proteins such as LIM kinases and CAP1 are not in abundance in the cell to promote the nucleotide exchange on actin monomers from ADP to ATP that is required for polymerization to take place, an overall decrease in cell migration is observed. Future studies should include knocking down WDR1 and WDR Δ 35 with siRNA in mammalian cells to determine how it affects actin filament turnover rates and cell migration. Moreover, it would be interesting to see whether overexpression of WDR Δ 35 is able to compensate for the loss of WDR1 in cells or vice versa.

Previous studies in our lab have shown that cofilin overexpression in HEK293 cells caused a significant upregulation in WDR Δ 35 levels as well as a down regulation in

WDR1 levels. Thus, WDR Δ 35 may be acting as a dominant negative by interacting with WDR1 and altering its conformation, which in turn lowers its binding affinity for cofilin. Overall, this could serve as a regulatory mechanism when cofilin levels are too high in the cell to prevent the overproduction of ADP-bound G-actin monomers that are incompetent for polymerization. Therefore, for another future direction cofilin could be overexpressed with WDR1 in HEK293 cells to examine the F/G actin ratios and determine whether there is a higher amount of F-actin in the cell which would strengthen the hypothesis that WDR Δ 35 is interacting with WDR1 to decrease or inhibit its interaction with cofilin to enhance its actin filament depolymerizing activity. In addition, it would be interesting to measure the levels of LIM kinase, phosphorylated LIM kinase (Ser-506/Ser-508), cofilin and phosphorylated cofilin (Ser-3) in cells that are overexpressed with WDR1 and or WDR Δ 35 to investigate whether any of their levels are changed in comparison to a normal control. This would confirm whether WDR1 and or WDR Δ 35 caused a decrease in cell migration rates due to effects on LIM kinase ability to phosphorylate cofilin and allow the release of ADP actin monomers that are incapable of polymerization.

Furthermore, although alone WDR1 and WDR Δ 35 appear to have redundant effects on actin filament turnover rates and cell migration rates when overexpressed in the cell, their effects could vary when they are both overexpressed simultaneously in the cell. For future directions I would like to overexpress both WDR1 and WDR Δ 35 in mammalian cells and then perform a co-immunoprecipitation (co-IP) experiment to determine whether they are interacting with one another. It would also be interesting to examine the actin turnover rates in these cells to establish whether there is less G-actin monomers in cells that are overexpressed with both WDR1 and WDR Δ 35 relative to cells

that are overexpressed with WDR1 or WDR Δ 35 alone. If indeed they are interacting with one another and there are less G-actin monomers in the supernatant of cells that are overexpressed with both WDR1 and WDR Δ 35 this would further strengthen the argument that WDR Δ 35 is acting as a dominant negative by interacting with WDR1 and lowering its affinity for cofilin. By doing so it would slow down the production of ADP-actin which is incompetent for polymerization.

Moreover, transfection of GFP-tagged WDR1 and WDR Δ 35 in HEK293 cells and immunoprecipitation with anti-GFP antibody revealed that both WDR1 and WDR Δ 35 do not interact with actin, CAP1 or cofilin under the lysis conditions applied. Therefore, for future directions I would like to repeat this experiment under different cell lysis conditions to determine whether specific cell conditions are required to establish interactions between WDR1/WDR Δ 35 and actin, cofilin, CAP1 or other potential binding partners such as profilin. Finally, alternative assays such as the Actin Binding Protein Spin-Down Assay Kit (Cytoskeleton Inc.) could be used to determine whether WDR1 and WDR Δ 35 bind to G-actin or F-actin in the cell. This co-sedimentation assay would also reveal if WDR1 or WDR Δ 35 are able to bundle or sever actin filaments.

Studies have shown that WDR1 is upregulated in breast cancer and ovarian cancer tissues where it may contribute to the observed enhancement in cell migration and invasion rates in these cells (Haslene-Hox, 2013; Kang, 2010). For future directions I would like to overexpress WDR1, WDR Δ 35 and all of the described mutants in different cancer cell lines such as MCF-7 and MDA-MB-231 cells which are common breast cancer cell lines, and investigate their effects on actin filament turnover rates and cell migration. It would be interesting to see if the overexpression of WDR1 or WDR Δ 35 in

cancer cells would have similar or different effects on actin turnover rates and cell migration rates in comparison to HEK293 cells and how the mutants may alter these effects. This would give valuable insight into ways to prevent malignant cancer cells from invading healthy tissues by targeting actin binding proteins that are involved in the dynamics and reorganization of the actin cytoskeleton during cell migration.

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Appendix A: Summary of the primers used to introduce point mutations in WDR1 and WDRΔ35

	FORWARD PRIMER	REVERSE PRIMER	SEQUENCING PRIMER
WDR1 MUTANTS			
WDRR17G	5'GCCAGCCTCCC CCAGGTGGAGGG GGGTGTCTCTAA G3'	3'TTCTTCCACAA GCGGTCGGAGGG GGTCCACCTC5'	3'GGGGGTAAGTT CAAGTTCAAATG TTAACCACTG5'
WDRH188Q	5'TTCAAGTTTAC AATTGGTGACCA AAGCCGCTTTGT C3'	3'GGGGGTAAGTT CAAGTTCAAATG TTAACCACTG5'	5'TTCAAGTTTAC AATTGGTGACCA AAGCCGCTTTGT C3'
WDRG299E	5'CTCCTTAGCAT CTCCCTGTCTGAA TACATCAACTAC3 ,	3'GTCTTCCTGGT GGAGGAATCGTA GAGGGACAGA5'	5'TTCAAGTTTAC AATTGGTGACCA AAGCCGCTTTGT C3'
WDRG344E	5'ATCTATTCTGG GAGCCATGATGA GCATATTAATTA C3'	3'CCGTTTtaggAT GTAGATAAGACC CTCGGTACTA5'	5'CTCCTTAGCAT CTCCCTGTCTGAA TACATCAACTAC3 ,
WDRH531Y	5'GAGAATAATGT TTTTTATGGATAT CATGCAAAAATT 3'	3'CTACCGATAAG CCTCTTATTACAA AAAATACCT5'	5'ATCTATTCTGG GAGCCATGATGA GCATATTAATTA C3'
WDRW602STOP	5'CATGACGCCTC TGTGAAGGAGTG AACAATCACCTA C3'	3'CAGTGGTGGAG GGTACTGCGGAG ACACTTCCTC5'	5'GAGAATAATGT TTTTTATGGATAT CATGCAAAAATT 3'
WDRΔ35 MUTANTS			
WDRΔ35H48Q	5'ATCCTAAGGAA CATCGACGACCA AAGCCGCTTTGT C3'	3'CCTTTCACGCA GTAGGATTCCCT GTAGCTGCTG5'	5'TTCAAGTTTAC AATTGGTGACCA AAGCCGCTTTGT C3'
WDRΔ35G159E	5'CTGCTCAGTGT CTCCCTGTCCGA ATACATCAACTA T3'	3'GTCTTCCTGGT GGACGAGTCACA GAGGGACAGG5'	5'ATCCTAAGGAA CATCGACGACCA AAGCCGCTTTGT C3'
WDRΔ35G204E	5'ATTTACTCTGG GAGCCACGACGA GCACATTAATTA C3'	3'CCGTTcaggAT GTAAATGAGACC CTCGGTGCTG5'	5'CTGCTCAGTGT CTCCCTGTCCGA ATACATCAACTA T3'

Appendix B: Summary of the point mutations in WDR1 and WDRΔ35

	NUCLEOTIDE CHANGE	AMINO ACID CHANGE	AMINO ACID NUMBER	WD REPEAT REGION
WDR1 MUTANTS				
WDRR17G	CGG→GGG	Arg (R) → Gly(G)	17	
WDRH188Q	CAC→CAA	His (H) → Gln(Q)	188	WD4
WDRG299E	GGA→GAA	Gly (G) → Glu(E)	299	
WDRG344E	GGC→GAG	Gly (G) → Glu(E)	344	WD6
WDRH531Y	CAC→TAT	His (H) → Tyr (Y)	531	WD8
WDRW602STOP	TGG→TGA	Try (W) → STOP	602	WD9
WDRΔ35 MUTANTS				
WDRΔ35H48Q	CAC→CAA	His (H) → Gln(Q)	48	WD1
WDRΔ35G159E	GGG→GAA	Gly (G) → Glu(E)	159	
WDRΔ35G204E	GGA→GAG	Gly (G) → Glu(E)	204	WD3

VITA AUCTORIS

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B.Sc. in Biological Sciences with Thesis, 2011
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Microbiology (2013)
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